

Purification and biochemical characterization of a fibrin(ogen)olytic metalloprotease from *Macrovipera mauritanica* snake venom which induces vascular permeability

EUN HEE LEE, JUNG EUN PARK, JONG WOO PARK and JUNG SUP LEE

Department of Biomedical Science and BK21-Plus Research Team for Bioactive Control Technology,
College of Natural Sciences, Chosun University, Gwangju 501-759, Republic of Korea

Received April 8, 2014; Accepted July 21, 2014

DOI: 10.3892/ijmm.2014.1864

Abstract. In the present study, a novel fibrin(ogen)olytic metalloprotease from *Macrovipera mauritanica* snake venom was purified and characterized in terms of enzyme kinetics and substrate specificity. The purified enzyme [termed snake venom metalloprotease-*Macrovipera mauritanica* (SVMP-MM)] was composed of a single polypeptide with an apparent molecular weight of 27 kDa, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The N-terminus of the enzyme was composed of NH₂-QRFAPRYIEL-COOH, as determined by N-terminal sequencing. The A α - and the B β -chains of fibrinogen were completely cleaved by SVMP-MM within 20 and 480 min, respectively. However, the γ -chain was much more resistant to digestion by the enzyme. The enzyme also exhibited proteolytic activity, cleaving the α - α polymer of cross-linked fibrin, but did not effectively digest the γ - γ polymer. To determine the kinetic parameters for SVMP-MM, a fluorescence-quenching peptide (termed *o*-aminobenzoic acid-HTEKLVTS-2,4-dinitrophenyl-NH₂) containing a K-L

sequence for SVMP-MM cleavage was designed and synthesized. The optimal pH and temperature for the enzyme activity were found to be 5.5 and 37°C, respectively, when the fluorogenic substrate was synthesized and used as a substrate. Among the various divalent cations tested, Ni²⁺ and Cu²⁺ showed strong inhibitory effects on enzyme activity, with an average of 69.6% inhibition. The enzyme activity was also inhibited by treatment with 1,10-phenanthroline, ethylenediaminetetraacetic acid and glycol-*bis*-(2-aminoethylether)-N,N,N',N'-tetraacetic acid, but not with aprotinin, tosyl-lysine chloromethyl ketone and tosyl-phenylalanyl chloromethyl ketone, suggesting that SVMP-MM is a metalloprotease and not a serine protease. The enzymatic parameters, including the K_M , k_{cat} , and k_{cat}/K_M values were estimated to be 0.015 mM, 0.031 sec⁻¹, and 20.67 mM⁻¹sec⁻¹, respectively. SVMP-MM induced vascular permeability by digesting type IV collagen. The results obtained in our study demonstrate that SVMP-MM is a fibrin(ogen)olytic P-I class metalloprotease, which can induce a hemorrhagic reaction *in vivo*.

Correspondence to: Professor Jung Sup Lee, Department of Biomedical Science, College of Natural Sciences, Chosun University, 309 Pilmun-daero, Dong-gu, Gwangju 501-759, Republic of Korea
E-mail: jsplee@chosun.ac.kr

Abbreviations: 1,10-PT, 1,10-phenanthroline; Abz, *o*-aminobenzoic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DMF, dimethylformamide; Dnp, 2,4-dinitrophenyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FV, factor V; EGTA, glycol-*bis*-(2-aminoethylether)-N,N,N',N'-tetraacetic acid; FX, factor X; FXIIIa, factor XIIIa; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; *p*NA, *para*-nitroaniline; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; serpin, serine proteinase inhibitors; TEMED, tetramethylethylenediamine; TLCK, tosyl-lysine chloromethyl ketone; TPCK, tosyl-phenylalanyl chloromethyl ketone; XL-fibrin, cross-linked fibrin.

Key words: snake venom, *Macrovipera mauritanica*, metalloprotease, snake venom metalloprotease-MM, fibrin(ogen)olytic enzyme, vascular permeability

Introduction

Snake venom contains a variety of enzymes and peptides that help the snake to overpower, kill, and/or digest its prey (1-3). As a result, envenomation following a snake bite evokes serious consequences, including systemic bleeding, myolysis, coagulopathy, hypovolemia, hemodynamic shock and acute renal failure, which are accompanied by a series of systemic deleterious effects in animals (1,2,4,5). Among the snake venom components, a variety of proteolytic enzymes are directly or indirectly involved in evoking the symptoms as preliminary factors (1,3,4). Proteases are basically classified into snake venom serine proteases (SVSPs) and snake venom metalloproteases (SVMPs), depending on their sensitivity to inhibitors (3,5).

Recent proteomic analyses of snake venom have demonstrated that SVMPs are the major components in venom and constitute 11% to >65% of the total venom protein contents (6). In fact, SVMPs are directly involved in the induction of local and systemic hemorrhaging (1,7). The proteases degrade the components of basement membranes underlying capillary endothelial cells (8). Thus, they trigger the disruption of the blood vessel wall and eventually evoke the release of the blood

contents into the stroma (1,7). Studies have indicated that some SVMPs have fibrin(ogen)olytic activity (6,9), while others serve as prothrombin activators (10) or inactivators against blood serpins (11), demonstrating that the enzymes play pleiotropic roles in the disturbance of the hemostatic system and the induction of the leakage of blood components (6).

Macrovipera mauritanica (*M. mauritanica*; common name, Moorish viper) is a venomous viper found in north-western Africa and one of four species comprising the genus *Macrovipera*, together with *M. deserti*, *M. lebetina* and *M. schweizeri* (12). To date, however, a little is known about the proteases in their venom. As regards the *M. deserti* and *M. lebetina*, to the best of our knowledge, there are only two studies available which have examined their venom; one study examined the hemorrhagic, necrotizing and inflammatory-edematogenic activity (13), and the other surveyed para-specific neutralization against the venom using a polyvalent serum (14). *M. lebetina* venom, however, has been relatively well documented (15-17). The venom contains several SVSPs, such as β -F-genase and factor V (FV) activator, and also has SVMPs, including lebetases and factor X (FX) activator (5,18). The SVSPs and SVMPs all affect blood coagulation and hemostasis (4,15). In the case of *M. mauritanica*, to the best of our knowledge, there is no study available to date demonstrating any fibrin(ogen)ase(s) that are possibly contained in its venom.

SVMPs are classified as α - and β -fibrin(ogen)ases, according to their proteolytic preference toward the α - or β -chain of fibrin(ogen) (19). Although they can cleave fibrinogen actively, they cannot induce the release of fibrinopeptides or fibrin clot formation as thrombin does (20). In contrast to SVMPs, fibrin(ogen)olytic serine proteases favorably cleave the β -chain with lower activity to the α -chain and generally do not cleave the γ -chain (15,18).

In this study, a novel fibrin(ogen)olytic metalloprotease termed SVMP-*M. mauritanica* (SVMP-MM), was purified and characterized from the *M. mauritanica* snake venom. We describe the purification and characterization of the enzyme, with focus on its biochemical properties in terms of enzyme kinetics and substrate specificity toward a fluorogenic peptide (newly designed and synthesized in this study) and various blood coagulation-associated proteins, including plasminogen, fibrinogen and cross-linked (XL) fibrin. We also describe the effects of SVMP-MM on the induction of vascular permeability *in vivo*.

Materials and methods

Materials. Lyophilized *M. mauritanica* snake venom was purchased from Latoxan (Valence, France). All chromatographic columns, including Superdex 75 10/300 GL, Source 15Q 4.6/100 PE and Mono Q HR 5/5 were obtained from Amersham Biosciences Biotech Co. (Uppsala, Sweden). The PD-10 column was from Amersham Pharmacia Biotech Inc. (Uppsala, Sweden). Protein molecular weight markers were obtained from Fermentas (St. Leon-Rot, Germany). Human fibrinogen, α -thrombin, factor XIIIa (FXIIIa), glycol-bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline (1,10-PT), N,N'-methylene-bis-acrylamide, phenylmethanesulfonyl fluoride (PMSF), tetramethylethylenediamine (TEMED), Trizma base, and

other chemicals were purchased from Sigma (St. Louis, MO, USA). Polyvinylidene fluoride (PVDF) membranes were obtained from Bio-Rad (Hercules, CA, USA). Synthetic chromogenic substrates, such as Boc-LGR-*para*-nitroaniline (*p*NA) (typical substrate for FXa) and Boc-VPR-*p*NA (for thrombin) were from Seikagaku (Tokyo, Japan). Other chromogenic substrates, including H-D-VLK-*p*NA (S-2251 for plasmin), H-D-IPR-*p*NA (S-2288 for tPA), pyro-EGR-*p*NA (S-2444 for urokinase), MeO-Suc-RPY-*p*NA (S-2586 for chymotrypsin) and N- α -Z-D-RGR-*p*NA (S-2765 for FXa) were from Chromogenix (Milan, Italy). A fluorogenic peptide substrate [*o*-aminobenzoic acid (Abz)-HTEKLVTS-2,4-dinitrophenyl (Dnp)-NH₂ for SVMP-MM] was synthesized by GenScript (Piscataway, NJ, USA). KCl coagulometer was purchased from Sigma.

Purification of protease from snake venom. The lyophilized snake venom powder (121.8 mg) was dissolved in 1.5 ml of a standard buffer (20 mM sodium acetate, pH 5.5, 100 mM NaCl). To remove the insoluble materials, the crude venom solution was centrifuged at 9,000 \times g for 5 min and the resulting precipitate was discarded. The proteins (total 40.6 mg) contained in the supernatant were applied onto a Superdex 75 10/300 GL column equilibrated with standard buffer and then eluted with the same buffer at a flow rate of 0.5 ml/min. The active fractions were pooled and applied to a Source 15Q 4.6/100 PE column equilibrated with the same buffer. The bound proteins were eluted with a NaCl linear gradient ranging from 0 to 400 mM at a flow rate of 1.0 ml/min. The active fractions were pooled and desalted on a PD-10 column equilibrated with standard buffer. The desalted proteins were then loaded onto a Mono Q HR 5/5 column equilibrated with the same buffer. The bound proteins were eluted with a NaCl linear gradient ranging from 0 to 200 mM at a flow rate of 0.5 ml/min. The active fractions were pooled, desalted, concentrated and stored at -70°C as purified enzyme. In each purification step, protein concentrations were determined using Bradford reagent (Sigma). In addition, the fibrinogenolytic activity contained in each fraction was determined by fibrinogen clotting time (FCT) assay, slightly modified from the original thrombin clotting time (TCT) method (21). For the assay, 50 μ l of thrombin (2.5 U/ml) pre-incubated at 37°C for 5 min were mixed with 100 μ l of 5% fibrinogen, followed by the addition of various concentrations of protein samples to be tested with or without 1 mM EDTA. The clotting time was monitored using a KCl coagulometer. Protease activity was also examined using azocasein assay as previously described (22).

Proteolytic activity assay with a fluorogenic peptide substrate. The fluorogenic peptide substrate (termed Abz-HTEKLVTS-Dnp-NH₂) dissolved in 30% dimethylformamide (DMF) was resuspended in standard reaction buffer (20 mM sodium acetate, pH 5.5, 100 mM NaCl) at a final concentration of 80 μ M and incubated at 37°C during which the increase in fluorescence was monitored at $\lambda_{\text{ex}}=320$ nm and $\lambda_{\text{em}}=420$ nm for 15 min using a spectrofluorometer (Molecular Devices Corp., Sunnyvale, CA, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli (23). Protein samples to be analyzed were

mixed with an equal volume of 2X SDS-PAGE sample buffer, boiled at 100°C for 3 min and then loaded onto 8, 10 or 12% gels. Following electrophoresis, the protein bands were visualized by staining the gel with Coomassie brilliant blue. Protein molecular weight markers used for SDS-PAGE were as follows: β -galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction enzyme *Bsp*981 (25 kDa), β -lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa).

Fibrinogen cleavage assay. To examine the fibrinolytic activity of the purified enzyme, human fibrinogen (3.47 mg/ml) was dissolved in standard reaction buffer and incubated with SVMP-MM enzyme (5.5 mg/ml) at 37°C at a final volume of 140 μ l, in which the molar ratio of enzyme vs. fibrinogen was 1:50. From the reaction mixture, 15 μ l each of aliquots was withdrawn at various time periods and the reaction was terminated by the addition of 3 μ l of 6X SDS-PAGE sample buffer. The samples were then boiled for 3 min and the resulting products were analyzed by SDS-PAGE as previously described (22,24).

Turbidity assay for the spontaneous polymerization of fibrin monomers. The spontaneous polymerization of fibrin monomers generated from fibrinogen by thrombin or SVMP-MM cleavage was examined by measuring the increase in turbidity as previously described (22). Typically, 90 μ g of fibrinogen dissolved in standard reaction buffer were mixed with 0.02 units of thrombin, 0.04 units of plasmin or SVMP-MM enzyme (3 or 6 μ g) at 37°C and the increase in the absorbance at 350 nm was then recorded using a 96-well plate reader (Molecular Devices Corp.). The fibrinolytic activity of plasmin or SVMP-MM was determined by measuring the decrease in turbidity of XL-fibrin. Typically 90 μ l of 1 mg/ml fibrinogen in standard reaction buffer were added to 10 μ l of thrombin (17.7 U/ml) and pre-incubated for 1 h at 25°C to allow the formation of fibrin polymer. Thereafter, 10 μ l of SVMP-MM enzyme (3 or 6 μ g) or plasmin (0.04 or 0.08 units) were added and further incubated for 2 h at 37°C. The increase or decrease in absorbance at 350 nm was then recorded with a 96-well plate reader.

Analysis of the N-terminal sequence of the purified enzyme. The purified enzyme was subjected to electrophoresis on 12% SDS-polyacrylamide gel and transferred onto a PVDF membrane in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (pH 11) containing 10% methanol. The blot was stained with Coomassie brilliant blue, followed by destaining as previously described (24). A target band was excised from the membrane and subjected to N-terminal sequencing with a Precise 491 HT protein sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing was performed by the Korea Basic Research Institute (Daejeon, Korea).

Type IV collagen digestion. Type IV collagen (0.16 mg/ml) was incubated with SVMP-MM (40 μ g/ml) in 200 μ l of the standard reaction buffer at 37°C. At various time intervals, 30 μ l each of aliquots was withdrawn and mixed with 6 μ l of 6X SDS-PAGE sample buffer. The samples were boiled for 3 min and the proteins were then electrophoresed on 8% polyacrylamide gel, followed by staining the gel with Coomassie brilliant blue.

Effects of various protease inhibitors, divalent ions, pH and temperature on enzyme activity. To examine the effects of various inhibitors [EDTA, EGTA, dithiothreitol (DTT), tosyl-lysine chloromethyl ketone (TLCK), tosyl-phenylalanyl chloromethyl ketone (TPCK), 1,10-PT, bestatin, aprotinin and PMSF] or divalent ions (Ca^{2+} , Cu^{2+} , Fe^{2+} , Mg^{2+} , Mn^{2+} , Ni^{2+} and Zn^{2+}) on enzyme activity, reaction mixtures were composed of 3 μ g of purified enzyme, 1 mM of corresponding additive and 80 μ M of a fluorogenic peptide (Abz-HTEKLVTS-Dnp-NH₂) as a substrate in standard reaction buffer and incubated for 15 min at 37°C. The effects of temperature on enzyme activity were also assayed at different temperatures under the conditions described above. The pH dependency of the enzyme was also examined at 37°C under different buffer systems with the same concentrations of enzyme and fluorogenic peptide substrate. The buffer systems used for the pH requirements of the enzyme were as follows: 50 mM sodium acetate (pH 4.0-5.5); 50 mM potassium phosphate (pH 6.0-7.5); 50 mM Tris-HCl (pH 8.0-8.5); 50 mM glycine-NaOH (pH 9.0-10.5). In all cases, the reactions were triggered by the addition of 80 μ M of the fluorogenic peptide and the relative fluorescence units (RFUs) were monitored at $\lambda_{\text{ex}}=320$ nm and $\lambda_{\text{em}}=420$ nm for 15 min using a spectrofluorometer.

Vascular permeability assay. Vascular permeability induced by the enzyme was examined by a modification of the Miles assay (25). Evans blue dye solution was freshly prepared in 0.6% phosphate-buffered saline (PBS) at a final concentration of 5% and filtered through a paper (0.2 μ m in pore size) before use. A guinea pig (300 g in body weight, male) was lightly anesthetized with diethyl ether, and the dye (65 mg/kg body weight) was administered intravenously, followed by the intradermal injection of 10 μ g of SVMP-MM (dissolved in PBS) at the back of the animal. After 10 min, the guinea pigs were euthanized by urethane overdose and photographed to visualize the dye leakage. For the quantification of the dye leakage, the back skin around the injection spot (approximately 1 cm²) was cut out, soaked in 3 ml of formamide, and incubated for 48 h at 60°C to allow the release of the dye. The amount of dye exclusion was determined by measuring the absorbance at 620 nm and expressed as a measure in micrograms of Evans blue dye efflux, as previously described (25). All efforts were made to minimize animal suffering and to reduce the number of animals used. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, 1996.)

Results

Purification of a fibrin(ogen)olytic enzyme from *M. mauritanica* snake venom. A fibrin(ogen)olytic metalloprotease was purified by three chromatographic steps, using a size exclusion and two ion exchanger columns in order. For the purification, the crude venom powder of *M. mauritanica* (121.8 mg) was dissolved in standard buffer and centrifuged for 5 min at 9,000 x g to remove insoluble materials. The soluble proteins (total 40.6 mg) obtained were then fractionated by a size exclusion chromatography using a Superdex 75 10/300 GL column, from which the proteins were eluted at a flow rate of 0.5 ml/min (Fig. 1A). As shown in the elution profile of Fig. 1A, the proteins

Table I. Summary of the purification process for the SVMP-MM protease from *M. mauritanica* snake venom.

Purification step	Total protein (mg)	Total activity (U) ^a	Specific activity (U/mg)	Yield (%) ^b
Crude venom	121.8	19,500	160	100
Superdex 75	16.8	12,800	762	13.8
Source Q	4.2	3,360	800	3.5
Mono Q	2.8	2,800	1,000	2.3

^aOne unit (U) was defined as the amount of the enzyme digesting 80 μ M of fluorogenic peptide substrate termed Abz-HTEKLVTS-Dnp-NH₂ for 10 min. ^bTotal protein in the crude snake venom was assigned the value of 100%. SVMP, snake venom metalloproteases; Abz, *o*-aminobenzoic acid; Dnp, 2,4-dinitrophenyl.

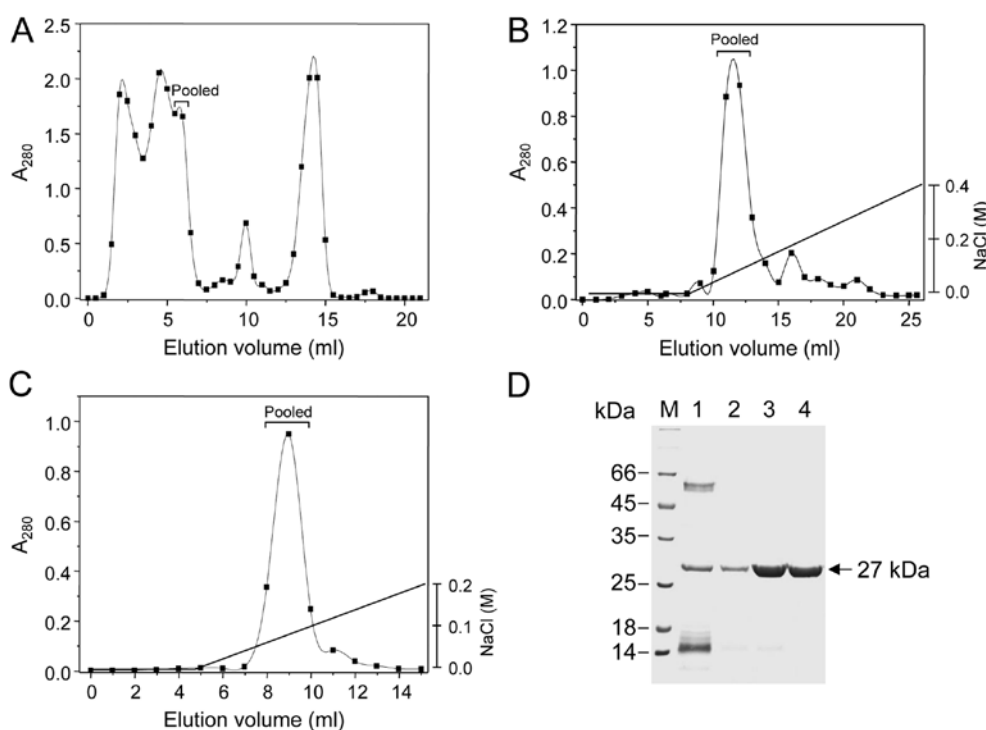


Figure 1. Purification of snake venom metalloprotease (SVMP) enzyme from *M. mauritanica* snake venom (SVMP-MM) using 3 chromatographic steps. (A) Size-exclusion chromatography. Crude snake venom (121.8 mg) was dissolved in standard buffer and the insoluble materials were removed by centrifugation for 5 min at 9,000 \times g. The resulting supernatant (total 40.6 mg of protein) was loaded on a Superdex 75 column and the proteins were eluted at a flow rate of 0.5 ml/min. Active fractions were pooled as indicated by the bracket. (B) Source Q column chromatography. The fractions pooled from the Superdex 75 column chromatography were applied to a Source column and the bound proteins were eluted with a NaCl linear gradient of 0 to 400 mM at flow rate of 1.0 ml/min. Active fractions were pooled as indicated by the bracket. (C) Mono Q column chromatography. Proteins collected from the Source Q column chromatography were applied to an anion exchanger Mono Q column and the proteins bound were eluted by a NaCl linear gradient of 0 to 200 mM at flow rate of 0.5 ml/min. In each chromatographic step, protein elution was monitored by measuring the absorbance at 280 nm. (D) SDS-PAGE of proteins obtained from each purification step. The proteins collected were electrophoresed on a 12% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. In each purification step, the fibrinolytic activity contained in each fraction was examined with fibrinogen clotting time (FCT) assay as described in Materials and methods. Lane M, protein molecular weight size makers; lane 1, crude snake venom; lane 2, proteins from Superdex 75 column; lane 3, proteins from Source Q column; lane 4, proteins from Mono Q column.

were separated into at least 5 peaks according to their sizes, among which the third one (12 to 14 in the elution volumes) showed fibrinolytic activity, as judged by FCT assay. From this column chromatography, a total of 16.8 mg proteins were obtained by pooling the 3 active fractions (Table I). The proteins acquired were further separated by an anion exchanger column chromatography on a Source 15Q 4.6/100 PE column (Fig. 1B). In this chromatography, the proteins bound were eluted with a NaCl linear gradient of 0 to 400 mM at a flow rate of 1.0 ml/min, from which 3 active fractions (10 to 12 in

the elution volumes) were pooled to acquire a total of 4.2 mg proteins (Fig. 1B and Table I). Thereafter, the proteins obtained were lastly applied and fractionated by another anion exchanger Mono Q HR 5/5 column chromatography. The proteins bound were eluted with a NaCl linear gradient ranging from 0 to 200 mM at flow rate of 0.5 ml/min, and 4 active fractions (7 to 10 in the elution volumes) were pooled (Fig. 1C). From this last chromatography, a total 2.8 mg of proteins could be obtained as a purified enzyme (Table I). The purified enzyme was designated to SVMP-MM. Table I summarizes the puri-

Table II. Comparison of the N-terminal amino acid sequence of SVMP-MM with those of several snake venom proteases.

Snake species	Enzyme	N-terminal sequence ^a	Identity (%)	Position of sequence	Accession no.
<i>M. mauntanica</i>	SVMP-MM	--QRFAPRYIEL	100	1-10	This study
<i>B. asper</i>	BAP1	-- ERFSP -YIEL	77.8	192-202	P83512
<i>M. lebetina</i>	Lebetase-II	--QRF E PRYIEL	90.0	195-205	Q98995
<i>M. lebetina</i>	Lebetase-4	--QQRF D PRYIEL	90.0	15-25	Q3ZD74
<i>M. lebitina</i>	VIF	-- ERFAPRY IEL	90.0	1-10	P83255
<i>B. moojeni</i>	BmooMP α -I	---RF SP - H IEL	75.0	1-8	3GBO_A
<i>B. newwiedi</i>	Neuwiedase	QQ R RF F Q R YIEL	60.0	1-12	Q919R4

^aN-terminal sequences located in fully processed proteases. Amino acid residues different from those of the SVMP-MM sequence are written in bold. The character '-' denotes the absence of an amino acid. SVMP, snake venom metalloprotease.

fication results. The specific activity of purified enzyme was 1,000 U/mg proteins and approximately 2.8 mg of enzymes were obtained in homogeneity, with 2.3% in yield (Table I).

Estimation of molecular weight and N-terminal amino acid sequence of SVMP-MM. The purified SVMP-MM enzyme appeared as single bands on an SDS-polyacrylamide gel stained with Coomassie brilliant blue and the molecular mass was estimated to be 27 kDa (Fig. 1D), a size similar to the metalloproteases, BlaH1 (28 kDa) from *Bothrops lanceolatus* (*B. lanceolatus*) (26) and VIF (26 kDa) from *M. lebetina* venom (27). In addition, SVMP-MM formed just one peak when it was eluted through gel filtration on a Superdex 75 10/300 GL column (data not shown). These results suggest that SVMP-MM is a monomeric protease with a small size. The N-terminus of SVMP-MM was composed of NH₂-QRFAPRYIEL-COOH, as analyzed by amino acid sequencing. The comparison of amino acid sequences showed that the N-terminal sequence of SVMP-MM was highly conserved in several metalloproteases derived from snake venoms, in which BAP1 from *Bothrops asper* (28), BmooMP α -I from *Bothrops moojeni* (29), neuwiedase from *B. newwiedi* (30), and 3 proteases (VIF, lebetases-II and -4) from *M. lebetina* (16,27,31) were included and there was an average of 80.5% identity between them (Table II).

Fibrinolytic and fibrinolytic activities of SVMP-MM. Various plasma proteins, including fibrinogen, plasminogen and prothrombin, which are involved in blood coagulation and hemostasis, were digested by the SVMP-MM enzyme (data not shown). However, there were no detectable plasmin and thrombin activities when the plasminogen and prothrombin were incubated with SVMP-MM in the presence of S-2251 (typical substrate for plasmin) and Boc-VPR-pNA (for thrombin) (data not shown). These results suggest that SVMP-MM can neither activate plasminogen nor prothrombin, as other SVMPs can (6,32). Fibrinogen was also one of efficient protein substrates for SVMP-MM (Fig. 2). The enzyme actively digested the A α - and the B β -chains of fibrinogen to a different extent (Fig. 2A). The A α - and the B β -chains of fibrinogen were completely degraded by the enzyme within 20 and 480 min, respectively. However, the γ -chain was much more resistant to digestion by the enzyme, even with overnight incubation (Fig. 2A). It is well known that

fibrin monomers generated from thrombin-cleaved fibrinogen can be polymerized spontaneously in the absence of FXIIIa *in vitro*, accompanied with an increase in turbidity as the polymerization proceeds (24). In this study, the spontaneous polymerization of fibrin monomers generated by thrombin or possibly SVMP-MM fibrinogen cleavage was examined by measuring the increase in turbidity. As expected, thrombin increased the turbidity effectively, but SVMP-MM did not. The thrombin-induced increase in turbidity was decreased by plasmin since the fibrin polymers were cleaved by the enzyme (Fig. 2B). As with plasmin, SVMP-MM decreased the thrombin-induced increase in turbidity in a dose-dependent manner. These results suggest that SVMP-MM cannot allow the fibrin monomers to be polymerized spontaneously when it cleaves fibrinogen. SVMP-MM was also able to cleave the XL-fibrin formed by thrombin in the presence of FXIIIa and fibrinogen (Fig. 2C). The enzyme showed α - α polymer-cleaving activity, accompanied with the generation of fibrin degrading products (FDPs); however, it could not digest γ - γ polymers effectively (Fig. 2C), as in the case of its fibrinogen cleavage (Fig. 2A). This XL-fibrin-cleaving activity of SVMP-MM was also examined by turbidity assay (Fig. 2D). Plasmin clearly decreased the turbidity of XL-fibrin in a dose-dependent manner, but SVMP-MM did not at the doses used. These results seem to be related to the inability of SVMP-MM in cleaving both the γ -chain of fibrinogen and the γ - γ polymers of XL-fibrin (Fig. 2D). SVMP-MM inhibited the thrombin-induced clotting time in a dose-dependent manner, as judged by FCT assay (Fig. 3). The clotting time was prolonged 2.2-fold, compared to that of the untreated control; however, the delaying effect of the enzyme was diminished in the presence of 1 mM of EDTA (Fig. 3), suggesting that SVMP-MM can delay thrombin-induced clotting time through a defibrinogenation and this is related directly to the enzyme activity. The results presented in Figs. 2 and 3 suggest that SVMP-MM belongs to the fibrin(ogen)ase family which has no clot formation activity, as it can just cleave predominantly on the A α -chain and less on both the B β - and the γ -chains of fibrinogen subunits (33,34).

Design and synthesis of a fluorescence-quenching peptide substrate for SVMP-MM. In this study, a fluorescence-quenching peptide substrate was designed and synthesized on the basis

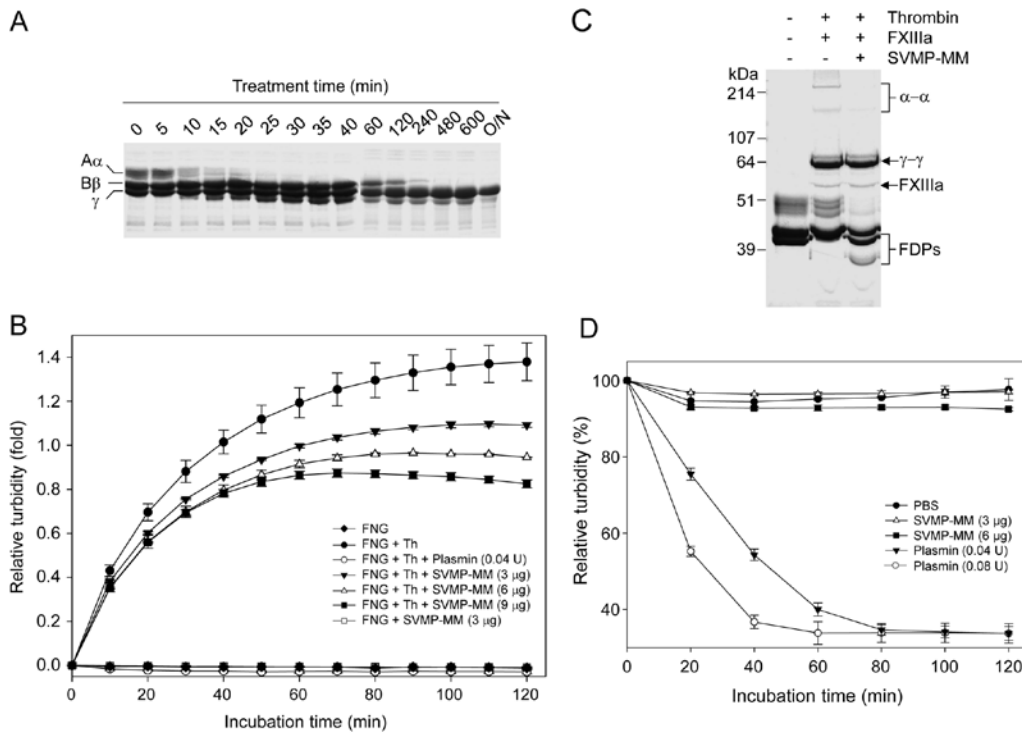


Figure 2. Fibrinolytic activity of the enzyme from snake venom metalloprotease (SVMP) enzyme from *M. mauritanica* snake venom (SVMP-MM). (A) Fibrinogenolytic activity assay. SVMP-MM enzyme was added to fibrinogen solution at a molar ratio of 1:50 and the reaction mixture was incubated for various time periods at 37°C as indicated. The reaction products were electrophoresed on 10% polyacrylamide gel and stained with Coomassie brilliant blue. The positions of A α -, B β - and γ -chains of fibrinogen are shown on the left side of the panel. O/N denotes overnight incubation at 37°C. (B) Determination of the spontaneous polymerization of fibrin monomers. Fibrinogen (90 μ g) dissolved in the standard reaction buffer (pH 5.5) was mixed with thrombin (0.02 units) or SVMP-MM (3 or 6 μ g) at 37°C. The increase in turbidity was monitored at 350 nm and expressed as relative activity as a percentage as described in Materials and methods. FNG, fibrinogen; Th, thrombin. (C) Fibrinolytic activity assay. The cross-linked (XL)-fibrin formed from fibrinogen (0.7 mg/ml) by thrombin (0.13 U/ml) in the presence of factor XIIIa (FXIIIa) (0.13 U/ml) and 1 mM CaCl₂ was incubated with 2 μ g of SVMP-MM enzyme for 30 min at 37°C. The reaction products were electrophoresed on an 8% SDS-polyacrylamide gel and stained with Coomassie brilliant blue to visualize proteins. The '+' and '-' symbols represent the addition and the omission of the corresponding additive, respectively. The positions of α - α and γ - γ chains of XL-fibrin are shown on the right side of the panel. FDPs, fibrin degrading products. (D) Determination of the fibrinolytic activity of plasmin and SVMP-MM by turbidity assay. Fibrinogen (90 μ g) dissolved in the standard reaction buffer, pH 5.5) was mixed with thrombin (0.18 units) and pre-incubated for 1 h at 25°C to allow the formation of fibrin polymer. SVMP-MM (3 or 6 μ g) or plasmin (0.04 or 0.08 units) was then added and further incubated for 2 h at 37°C, during which the decrease in absorbance at 350 nm was then recorded with a 96-well plate reader.

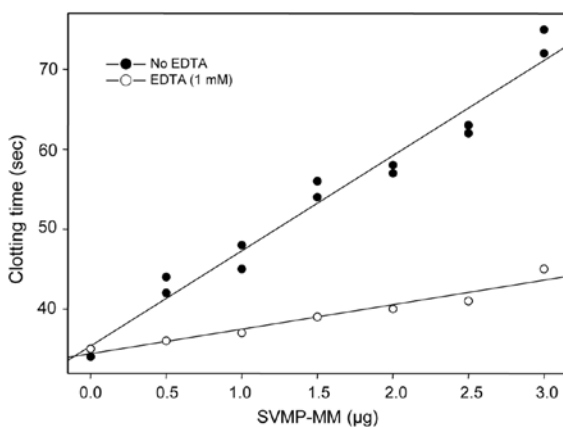


Figure 3. Fibrinogen clotting time (FCT) assay. Various concentrations of snake venom metalloprotease (SVMP) enzyme from *M. mauritanica* snake venom (SVMP-MM) were incubated with thrombin (0.125 units) and fibrinogen (0.3%) in the absence (●) or presence (○) of 1 mM ethylenediaminetetraacetic acid (EDTA) for 5 min at 37°C. FCT values were then monitored using a KC1 coagulometer and expressed as clotting time in seconds (sec).

of the cleavage site of SVMP-MM on its protein substrate. To locate the cleavage site of the enzyme on the fibrinogen

substrate first, 140 μ g of fibrinogen were digested with 1 μ g of enzyme for 20 min at 37°C and the peptide products generated were separated on a 12% SDS-polyacrylamide gel (Fig. 4A). Among the peptide fragments produced, a distinct 17.5 kDa protein band was excised from the gel and its N-terminus was sequenced. The sequencing results showed that SVMP-MM specifically cleaved the peptide bond of Lys⁴¹³ and Leu⁴¹⁴, which is located in the α -chain of fibrinogen (35). On the basis of this result, a peptide flanked by the Abz and Dnp groups, namely, Abz-HTEKLVTS-Dnp-NH₂ containing SVMP-MM cleavage site (K-L) was designed and synthesized in order to form a fluorescent donor (Abz group)-acceptor (Dnp group) pair. Therefore, Abz fluorescence would be quenched by the Dnp group (36) until the enzyme cleaves the peptide bond located between K and L residues in the synthetic peptide. The RFU clearly increased in a dose-dependent manner, when SVMP-MM (3 μ g) was incubated with various concentrations of the fluorogenic substrate and then the fluorescence produced was monitored for 15 min at λ_{ex} = 320 nm and λ_{em} = 420 nm (Fig. 4B). This result suggests that the fluorescence-quenching peptide synthesized may be used as a suitable substrate for elucidating the enzymatic properties of SVMP-MM.

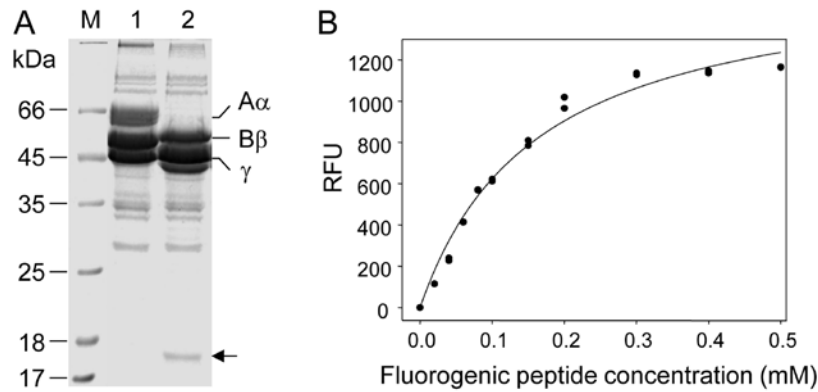


Figure 4. Cleavage site and fluorogenic activity of snake venom metalloprotease (SVMP) enzyme from *M. mauritanica* snake venom (SVMP-MM). (A) Fibrinogen cleavage by SVMP-MM. The enzyme (1 μ g) was incubated with 140 μ g of fibrinogen (FNG) at 37°C for 20 min and the products generated were then separated on 12% SDS-polyacrylamide gel, followed by staining with Coomassie brilliant blue to visualize. The positions of A α -, B β - and γ -chains of fibrinogen are shown on the right side of the panel. The arrow on the bottom of gel indicates a peptide fragment used for N-terminal sequencing. M, Protein molecular weight size makers; lane 1, FNG only; lane 2, FNG plus SVMP-MM. (B) Fluorogenic activity of SVMP-MM towards *o*-aminobenzoic acid (Abz)-HTEKLVTS-2,4-dinitrophenyl (Dnp)-NH₂. SVMP-MM (3 μ g) was incubated with various concentrations of fluorogenic peptide substrate for 10 min at 37°C and the fluorescence produced was monitored for further 15 min at λ_{em} =420 nm and λ_{ex} =320 nm. The level of fluorescence emitted was expressed as the relative fluorescence unit (RFU).

Table III. Effects of various divalent cations, protease inhibitors and chemical reagents on SVMP-MM activity.

Additive	Concentration (mM)	Relative activity (%) ^a
Control	0	100
Ca ²⁺	1	95.1
Cu ²⁺	1	17.6
Fe ²⁺	1	88.6
Mg ²⁺	1	98.2
Mn ²⁺	1	104.1
Ni ²⁺	1	43.2
Zn ²⁺	0.1	95.8
Zn ²⁺	1	78.5
TLCK	1	103.5
TPCK	1	99.7
EDTA	1	0
1,10-PT	1	1.2
Bestatin	0.01	81.6
PMSF	1	92.6
Aprotinin	1	97.9
EGTA	1	3.1
DTT	1	0

^aA synthetic fluorogenic peptide (Abz-HTEKLVTS-Dnp-NH₂) was used for the SVMP-MM activity assay as a substrate with or without the corresponding additive at 37°C for 15 min in standard buffer as described in Materials and methods. The RFU value from the untreated control was assigned 100%. SVMP, snake venom metalloprotease; RFU, relative fluorescence unit; TLCK, tosyl-lysine chloromethyl ketone; TPCK, tosyl-phenylalanyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; 1,10-PT, 1,10-phenanthroline; PMSF, phenylmethanesulfonyl fluoride; EGTA, glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

were examined with the fluorescence-quenching peptide, Abz-HTEKLVTS-Dnp-NH₂, as a substrate (Fig. 5). The enzyme exhibited its optimum pH (Fig. 5A) and temperature (Fig. 5B) at 5.5 and 37°C, respectively, with a total loss of activity under pH 10.5 and at 4°C. Considering the pH, temperature and ionic strength (data not shown) for the maximal enzyme activity, a standard reaction buffer consisting of 20 mM sodium acetate (pH 5.5) and 100 mM NaCl was prepared and used for further experiments. The effects of various divalent cations and protease inhibitors on SVMP-MM activity were examined (Table III). Divalent cations, such as Ca²⁺, Mg²⁺ and Mn²⁺ showed no significant stimulatory or inhibitory effects on SVMP-MM activity; however, Fe²⁺ and Zn²⁺ (1 mM) exhibited a slight inhibitory effect on the enzyme activity at different levels. However, a low dose of Zn²⁺ (<0.1 mM) did not show such a significant inhibitory effect on the activity (Table III). In addition, Ni²⁺ and Cu²⁺ showed relatively strong inhibitory effects on enzyme activity, with an average of 69.6% inhibition, compared to those of the untreated control. More importantly, the enzyme activity was clearly inhibited by treatment with 1,10-PT, EDTA and EGTA, which are all known metalloprotease inhibitors (37). Among the metalloprotease inhibitors used, bestatin did not act as an effective inhibitor against the enzyme activity. However, TLCK, TPCK, PMSF and aprotinin, which are typical serine protease inhibitors (5), showed no significant inhibitory effects on the enzyme activity (Table III). These results suggest that SVMP-MM is a typical metalloprotease requiring a certain metal ion as a co-factor for the enzymatic activity and not a serine protease. In addition, the enzyme activity was completely abolished by treatment with a reducing agent, such as DTT (Table III). This result suggests that a disulfide bond(s) located in the enzyme plays an important role in maintaining the enzyme activity. On the other hand, the enzyme kinetic parameters for SVMP-MM were also estimated using the fluorescence-quenching peptide as a substrate (Table IV). The K_M and k_{cat} values for the enzyme were estimated to be 0.015 mM and 0.031 sec⁻¹, respectively, when Abz-HTEKLVTS-Dnp-NH₂ was used as a substrate (Table IV). The k_{cat}/K_M value

Enzymatic properties and kinetic parameters of SVMP-MM.
The optimal pH and temperature for SVMP-MM activity

Table IV. Kinetic parameters for the cleavage of a fluorogenic peptide substrate by SVMP-MM.

Substrate used	K_M (mM) ^a	K_{cat} (sec ⁻¹) ^a	K_{cat}/K_M (mM ⁻¹ sec ⁻¹)
Abz-HTEKLVTS(Dnp)-NH ₂	0.015±0.009	0.31±0.042	20.67

^aData from six independent experiments were expressed as mean value ± SD. Abz, *o*-aminobenzoic acid; Dnp, 2,4-dinitrophenyl; SD, standard deviation.

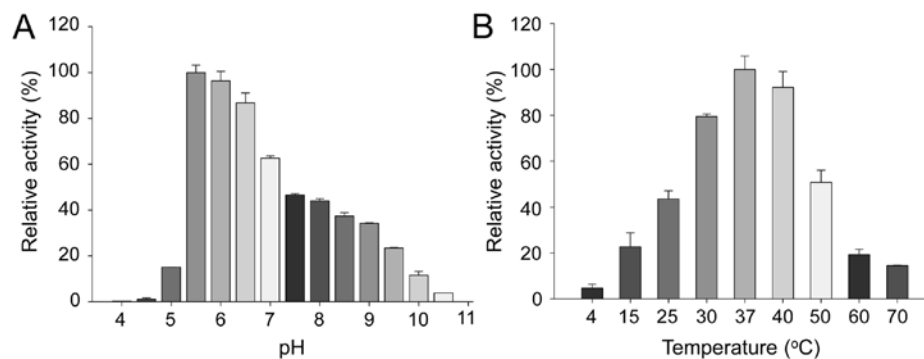


Figure 5. The pH- and temperature-dependency of snake venom metalloprotease (SVMP) enzyme from *M. mauritanica* snake venom (SVMP-MM). (A) Effect of pH on SVMP-MM activity. The enzyme (3 μ g) was incubated with the fluorogenic peptide [80 μ M of *o*-aminobenzoic acid (Abz)-HTEKLVTS-2,4-dinitrophenyl (Dnp)-NH₂ in a final concentration] for 30 min at 37°C under different pH conditions as described in Materials and methods. (B) Effect of temperature on the enzyme activity. The same amount of enzyme was incubated with the same amount of fluorogenic peptide for 30 min at various temperatures as indicated in the standard reaction buffer (pH 5.5). The fluorescence produced was monitored for further 15 min at λ_{em} = 420 nm and λ_{ex} = 320 nm. The level of fluorescence emitted was expressed as the reactive activity in percent, in which the fluorescence unit (RFU) values obtained in pH 5.5 and 37°C were regarded as 100%.

of SVMP-MM was found to be 20.67 mM⁻¹sec⁻¹, as well with the same fluorogenic substrate (Table IV).

Induction of vascular permeability and digestion of type IV collagen by SVMP-MM. In general, vascular permeability is a leakage of fluids and molecules from the blood stream to the extravascular space when the vascular basement membrane comprised of various protein components, such as laminin and type IV collagen is destroyed under a circumstance (38). This leakage can also be enhanced by effectors, such as inflammatory mediators and vascular endothelial growth factor (VEGF) (39). In this study, the activity of SVMP-MM related to the induction of vascular permeability was also observed *in vivo* (Fig. 6) using Miles assay as described in the Materials and methods, and as previously described (8,25). As shown in Fig. 6A-C, PBS only did not evoke the leakage of Evans blue dye (Fig. 6A); however, SVMP-MM induced vascular permeability (Fig. 6B), during which approximately 8 μ g of dye leakage occurred following the injection of 10 μ g of SVMP-MM (Fig. C). In addition, the enzyme digested type IV collagen, which is a major component of the vascular basement membrane (Fig. 6D). These results suggest that the induction of vascular permeability may be related to the SVMP-MM activity capable of digesting some basal membranes and extracellular matrix proteins, such as type IV collagen (32,39).

Discussion

In this study, a novel fibrin(ogen)olytic metalloprotease termed SVMP-MM from the *M. mauritanica* snake venom was purified

and biochemically characterized in terms of enzyme kinetics and substrate specificity. The enzyme was purified homogeneously by three chromatographic steps, including one size exclusion and two sorts of anion exchangers in order (Fig. 1). The purified SVMP-MM is a monomeric protease and 27 kDa in size, as judged by SDS-PAGE (Fig. 1D) and a gel filtration (data not shown). The size of SVMP-MM seems to be similar to that of several snake venom metalloproteases, such as BlaH1 (28 kDa) (26) and VIF (26 kDa) (27). In addition, the SVMP-MM has an amino acid stretch (that is N-QRFAPRYIEL-C) at its N-terminus, which shows high homology with those of metalloproteases, including BAP1 (28), lebetase-II, lebetase-4 and VIF (16,17,27,31,40) (Table II).

SVMP-MM actively digested various plasma proteins, including fibrinogen, plasminogen, and prothrombin, which are participating in blood coagulation and hemostasis. However, the enzyme did not show any amidolytic activity towards typical chromogenic substrates for plasmin (S-2251) and for thrombin (Boc-VPR-pNA). In addition, there was no occurrence of spontaneous polymerization of fibrin monomers, possibly produced from the fibrinogen cleavage by SVMP-MM, as judged by turbidity assay (Fig. 2B). These results demonstrate that the enzyme does not act as a plasminogen or prothrombin activator, as do other SVMPs from snake venom (6,32,41).

It should be noted that the A α -chain of fibrinogen was completely digested by the SVMP-MM protease for 20 min, while the B β - and the γ -chains remained intact for the same incubation time (Fig. 2A). As regards the B β -chain of fibrinogen, it was completely digested after 480 min of incubation. However, the γ -chain and the γ - γ polymer of fibrin(ogen)

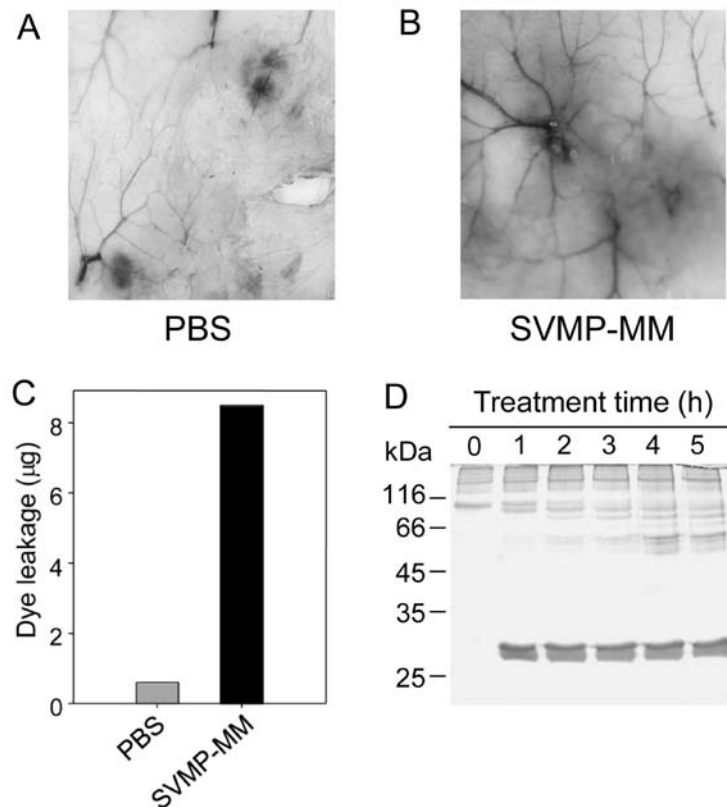


Figure 6. Induction of vascular permeability by snake venom metalloprotease (SVMP) enzyme from *M. mauritanica* snake venom (SVMP-MM). (A and B) Induction of vascular permeability by (A) phosphate-buffered saline (PBS) and (B) SVMP-MM. Evans dye was administered intravenously and then PBS or 10 µg of SVMP-MM was injected intradermally as described in Materials and methods. After 10 min, the animal was sacrificed and photographed. (C) Quantification of the dye leakage. To quantify the leakage of the dye, the back skin around the injection spot was cut out and then incubated in 3 ml of formamide at 60°C for 48 h. The dye exclusion was determined by measuring the absorbance at 620 nm and expressed as a measure in micrograms of Evans dye efflux. (D) Digestion of type IV collagen by SVMP-MM. Type IV collagen (total 32 µg) was incubated with 8 µg of SVMP-MM in 200 µl of the standard reaction buffer at 37°C. At various time intervals as indicated, 30 µl each of aliquots were withdrawn and mixed with 6 µl of 6X SDS-PAGE sample buffer. The samples were then boiled for 3 min and then the proteins were electrophoresed on 8% SDS-polyacrylamide gel, followed by staining the gel with Coomassie brilliant blue.

were not completely digested by the enzyme (Fig. 2A and D). A number of SVMPs, including fibrolase from *Agkistrodon contortrix contortrix* (*A. contortrix contortrix*) (42), atroxase from *Crotalus atrox* (*C. atrox*) (9) and lebetase from *M. lebetina* (43) preferentially cleave the A α -chain and the B β -chain of fibrinogen slowly, together with their digesting activities which favor towards the α - α polymer of fibrin. However, they cannot cleave the γ -chain and the γ - γ polymer of fibrin(ogen), apart from atrolysin F from *C. atrox* (9). Therefore, SVMP-MM seems to be a fibrin(ogen)ase belonging to snake venom metalloprotease family, which can digest both the A α - and the B β -chains of fibrinogen.

Azocasein assay is routinely used for examining the proteolytic activity of most proteases (22,24). Unfortunately, SVMP-MM did not effectively cleave azocasein as a substrate. Therefore, the assay seemed not to be suitable for examining SVMP-MM activity. The enzyme also showed only a background level of amidolytic activity against various chromogenic substrates listed in the Materials and methods. This inability of the amidolytic activity of the enzyme towards the chromogenic substrates may be due to its ability to cleave the peptide bond only at the amino side (termed forward-amino acid cleaving activity), rather than at the carboxyl side of the

peptide substrate. Since the chromogenic substrates used in this study are all coupled to pNA groups at their carboxyl sides, an amino acid-tethered pNA would be released by the enzyme action, which makes no measurable yellowish color change. For example, one protease of SVMPs from *B. moojeni* venom shows the forward-serine cleaving activity, accordingly the enzyme activity can be estimated only with a fluorescence-quenching peptide substrate MCA-GXXPSXQED-Dnp (29). Thus, it was absolutely necessary to obtain a sensitive peptide substrate for SVMP-MM, which could be used for investigating the pH- and temperature-dependencies, the responsiveness to various protease inhibitors and salts, and the determination of kinetic parameters. One way to prepare a peptide substrate for SVMP-MM was to synthesize it on the basis of the cleavage site of enzyme. Since SVMP-MM specifically cleaved the K-L bond located in the α -chain of fibrinogen substrate (Fig. 2A), an octameric fluorescence-quenching peptide containing the K-L cleavage site was synthesized, to which Abz (as a fluorescent donor) and Dnp (as a quencher) groups were attached at the N- and the C-termini, respectively (36,44). The SVMP-MM activity was sensitively assayed with this fluorescence-quenching peptide as a substrate by monitoring the increase in RFU at λ_{ex} = 320 nm and λ_{em} = 420 nm (Fig. 2B). The results

revealed that the fluorescence-quenching peptide synthesized may be used for effectively testing the activity of SVMP-MM from crude venom.

SVMP-MM showed its maximum activity at 37°C and under pH 5.5 (Fig. 5). Although one type of SVMP, such as CcHaseII from *Cerastes cerastes* also shows the maximal activity at the same conditions (45), similar to SVMP-MM, the relative weak acidic pH requirement for the SVMP-MM activity seems to be a slightly different characteristic from other SVMPs that exhibit optimal activities, in general, under neutral or weak alkalic conditions (16,47,48). The enzyme activity of SVMP-MM was greatly decreased at low temperatures (approximately 4°C) (Fig. 5B). This cold sensitivity of the enzyme was not expected as the majority of SVMPs, such as CcHaseII (45) and BpirMP (46) are still active at this temperature. The enzyme activity of SVMP-MM was inhibited by Zn²⁺ at 1 mM, and did not show significance at a low dose (Table III). This property is in accordance with those of some metalloproteases that contain Zn²⁺ in their catalytic centers (30). The enzyme activity of SVMP-MM was completely inhibited by a reducing agent DTT (Table III), demonstrating that a disulfide bond(s) located in the enzyme may play an important role in maintaining the enzyme activity. A number of SVMPs, including BAP1, lebetases, BmooMP α -I, and neuwiedase are also completely inhibited by DTT (30).

SVMPs are classified as P-I, P-II and P-III classes, depending on their size and domain structure differences (30). P-I proteases are small SVMPs in size (molecular masses of 20-30 kDa) and contain only a pro and a catalytic domain. P-II SVMPs are medium-sized enzymes (30-60 kDa) and are composed of pro, catalytic and disintegrin domains. P-IIIs are large enzymes (60-100 kDa) and contain pro, catalytic, disintegrin-like and cysteine-rich domains (29,30). A recent study on the crystal structures of P-I class SVMPs have demonstrated that SVMPs have a consensus HEXXHXXGXXHD sequence and a Met-turn structure that contains a conserved Met residue forming a hydrophobic basement for the three zinc-binding histidine residues in the consensus sequence (30). By these criteria, SVMP-MM can be categorized as a P-I class enzyme, as its molecular mass is 27 kDa composed of a single polypeptide (Fig. 1D) and its activity is completely inhibited by typical metalloprotease inhibitors, such as 1,10-PT and EGTA (Table III). However, further studies are required to reveal the domain structure of SVMP-MM and whether the enzyme has a catalytic domain containing a consensus Zn²⁺ binding motif and Met-turn structure, even though it has a high homology amino acid stretch at its N-terminus with those of typical P-I enzymes (Table II).

On the other hand, SVMP-MM has two typical activities: i) to induce a vascular permeability, as judged by Miles assay (Fig. 6B and C); and ii) to cleave type IV collagen (Fig. 6D), one of the basal membrane and extracellular matrix proteins, together with other proteins, such as fibronectin and laminin (32). Moreover, it also hydrolyzes plasma proteins, such as prothrombin, plasminogen, fibrinogen and XL-fibrin, which are involved in blood coagulation and hemostasis. Therefore, the ability of SVMP-MM to induce vascular permeability and degrade the plasma proteins and type IV collagen may likely be related to the disturbance of blood hemostasis by the enzyme. However, it cannot be ruled out that SVMP-MM

can induce vascular permeability by activating mast cells to release mediators, such as histamine, which provoke a rapid increase in plasma extravasation in animal skin (49). To verify the direct induction ability of SVMP-MM, further studies related to the activation of mast cells and the release of cellular mediators, such as histamine are required.

Taken together, the data from present study demonstrate that: i) SVMP-MM is a metalloprotease which is small in size (27 kDa molecular mass) from *M. mauritanica* snake venom (Fig. 1D); ii) it can cleave fibrinogen and XL-fibrin (Fig. 2) without having prothrombin- and prothrombin-activating abilities (data not shown); iii) it is very sensitive to divalent cation chelators, such as EDTA and EGTA and a reducing agent, such as DTT (Table III); iv) it can be inhibited by a typical metalloprotease inhibitor, such as 1,10-PT (Table III); v) it has a high sequence identity at its N-terminus to those of typical P-I SVMPs (Table II); and vi) it can induce vascular permeability by digesting type IV collagen (Fig. 6). In conclusion, these results demonstrate that SVMP-MM is a fibrin(ogen)olytic metalloprotease of the P-I class, which can induce a hemorrhagic reaction *in vivo*.

Acknowledgements

This study was supported by a research fund from Chosun University, 2013.

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