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cDNA cloning, expression and fibrin(ogen)olytic activity of two low-molecular weight snake venom metalloproteinases

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Abstract

Two cDNA clones, AplVMP1 and AplVMP2, were isolated from a snake (*Agkistrodon piscivorus leucostoma*) venom gland cDNA library. The full-length cDNA sequence of AplVMP1 with a calculated molecular mass of 46.61 kDa is 1,233 bp in length. AplVMP1 encodes PI class metalloproteinase with an open reading frame of 411 amino acid residues that includes signal peptide, pro-domain and metalloproteinase domains. The full-length cDNA of the AplVMP2 (1,371bp) has a calculated molecular mass of 51.16 kDa and encodes PII class metalloproteinase. The open reading frame of AplVMP2 with a 457 amino acid residues is composed of signal peptide, pro-domain, metalloproteinase and disintegrin domains. AplVMP1 and AplVMP2 showed 85% and 93% amino acid identical to PI class enzyme *Agkistrodon contortrix laticinctus* ACLPREF and PII class enzyme *Agkistrodon piscivorus piscivostatin*, respectively. When expressed in *E.coli*, most of recombinant proteins of AplVMP1 and AplVMP2 were in insoluble inclusion bodies, with soluble yields of 0.7 mg/l and 0.4 mg/l bacterial culture, respectively. Both affinity purified recombinant proteins show proteolytic activity on fibrinogen, although having an activity lower than that of crude *A.p.leucostoma* venom. Proteolytic activities of AplVMP1 and AplVMP2 were completely abolished after incubation with a final concentration of 100 μ m of EDTA or 1,10-phenanthroline. Both AplVMP1 and AplVMP2 were active in a fibrin-agar plate but devoid of hemorrhagic activity when injected (up to 50 μ g) subcutaneously into mice, and had no capacity to inhibit platelet aggregation.

Keywords

Snake Venom metalloproteinase; *Agkistrodon piscivorus leucostoma*; cDNA library; Fibrin(ogen)olytic activity

1. Introduction

Snake venoms, particularly those belonging to the Crotalidae and Viperidae families, are rich sources of metalloproteinases (Hite et al., 1994; Bjarnason and Fox 1994; Selistre de Araujo et al., 1997; Birkedal-Hansen, 1995). Snake venom metalloproteinases (SVMPs) are a superfamily of zinc-dependent proteases and need zinc or calcium atoms to work properly and degrade capillary basement membranes (Selistre de Araujo et al., 1995; Bjanason and Fox, 1994). Zinc metalloproteinases are widely occurring and participate in a number of

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important biological, physiological, and pathophysiological processes, such as hemorrhage, fertilization, thrombolysis, cancer metastasis, edema, hypotension, hypovolemia, inflammation and necrosis (Mori et al., 1984; Hooper, 1994; Fox and Serrano, 2005). SVMPs and 'A Disintegrin And Metalloproteinases' (ADAMs) constitute the Reprolysin subfamily (Hite et al., 1994; Bjarnason and Fox, 1994; Fox and Serrano, 2005).

On the bases of cDNA size, the diversity of biological activities and amino acid primary structure features, SVMPs were categorized into four classes (Hite et al., 1994; Bjarnason and Fox, 1994; Fox and Serrano, 2005): (1) PI class enzymes, the smallest reprolysin, contain a pro-domain and a metalloproteinase domain. A wide variety of PI enzymes with various functions have been isolated from different species of snakes, including leucurolysin-a (leuc-a) with fibrinogenolytic activity from *Bothrops leucurus* (Bello et al., 2006), BjussuMP- II with antiplatelet activity from *Bothrops jararacussu* (Marcussi, et al., 2007), BlaH1 with hemorrhagic, caseinolytic, fibrinogenolytic, collagenolytic and elastinolytic activities from *Bothrops lanceolatus* (Stroka et al., 2005), BaP1 with hemorrhage, myonecrosis, dermonecrosis, blistering and edema from *Bothrops asper* (Watanabe et al., 2003), BmooMP α -I with fibrinogenolytic activity from *Bothrops mooojeni* (Bernardes et al., 2008), as well as many others have been reported in the excellent review (Fox and Serrano, 2005). (2) PII class enzymes, the medium-size enzymes, comprise a pro-domain, a metalloproteinase and an disintegrin domain. To date, a number of PII class enzymes have been isolated from different species of snake such as Atrolysin with hemorrhage activity from *Crotalus atrox* (Hite et al., 1992; Jia et al., 1997), MT-d with proteolytic activity from *Agkistrodon halys brevicaudus* (Jeon and Kim, 1999), Bothrostatin precursor showing high inhibitory activity on collagen-induced platelet aggregation from *Bothrops jararaca* (Fernandez et al., 2005), and Albolatin with inhibiting collagen-induced platelet aggregation from *Trimeresurus albolabris* (Singhamatr and Rojnuckarin, 2007). (3) PIII class enzymes, the reprolysin and the most potent hemorrhagic toxins, have been synthesized with a pro-domain, a metalloproteinase domain, a disintegrin-like domain and an additional cysteine-rich domain. Numerous PIII class enzymes have been identified from different species of snakes, including Bothropasin with hemorrhagic and myonecrotic activities isolated from *Bothrops jararaca* (Assakura et al., 2003), metalloproteinase with proteolytic, edematogenic and myotoxic activities from *Bothrops alternatus* (Gay et al., 2005), BjussuMP-I with hemorrhagic and proteolytic activities from *Bothrops jararacussu* (Mazzi et al., 2004). (4) PIV class enzymes contain the non-processed PIII structure (a pro-domain, a metalloproteinase, a disintegrin-like, and a cysteine-rich domain) and two C-type lectin-like domains in the quaternary structure connected to the main chain of the PIII by disulfide bonds. To our knowledge, four PIV class enzymes have been isolated from different snakes, including RVV-X with an activation of Factor X to Xa from Russell's viper venom (Gowda et al., 1994; Chen et al., 2008), VLFXA, the Factor X activator from *Vipera lebetina* (Siigur et al., 2001, 2004), and VAFXA-I and VAFXA-II with the characteristics of hydrolyzing insulin B-chain, fibrinogen and some components of the extracellular matrix from *Vipera ammodytes ammodytes* (Leonardi et al., 2008). The crystal structure of RVV-X has recently been analyzed by Takeda et al. (2007).

Snake venom metalloproteinases play an important role in the digestion of prey tissue, participation in the pathophysiology of envenoming by inducing local and systemic bleeding, as well as other tissue-damaging activities and hemostatic alterations. Thus, these enzymes have been extensively studied, and research has focused on these compounds in the last few years mainly due to their pathological relevance (Gutiérrez and Rucavado, 2000; Rodrigues et al., 2004) and potential applications in therapeutics (Toombsb, 2001; Swenson et al., 2004), as well as their potential use as diagnostic, thrombolytic, apoptosis-inducing agents. Therefore, these enzymes merit further investigation. In this study, we isolated two cDNAs clones encoding two different classes of metalloproteinases, AplVMP1 and

AplVMP2, from a snake (*Agkistrodon piscivorus leucostoma*) venom gland cDNA library by random sequencing as described by Jia et al. (2008). The nucleotide sequences of AplVMP1 and AplVMP2 have been deposited into GenBank with accession numbers FJ429179 and FJ429180, respectively. Various functions of recombinant proteins of AplVMP1 and AplVMP2 were investigated.

2. Materials and methods

2.1. Plasmid construction

Polymerase chain reaction (PCR) was performed to amplify the full-length cDNAs encoding AplVMP1 and AplVMP2 using the PfuUltra Hotstart DNA Polymerase (Stratagene) and two pairs of primers. (1) AplVMP1F, TAAT GAATTCATGATCCAGGTTCTCTTGGTGA and AplVMP1R, ACT CTCGAGTCATCACGCCTCCAAAAGTTCATT. (2) AplVMP2F TAAT GAATTCATGATCCAAGTTCTCTTGGTGA and AplVMP2R ACT CTCGAGTCATTAGGCATGGAAGGGATTTCA. Two restriction enzyme sites: *EcoR* I in forward primer and *Xho* I in reverse primer were introduced (boxed sequence). Clones 01E11 accession number FJ429179 for AplVMP1 and 20F10 accession number FJ429180 for AplVMP2 from a cDNA library (Jia et al., 2008) were separately used as PCR templates. PCR was performed using a thermal cycler (Gene Cyclor, BIO-RAD Hercules, CA, USA) programmed for an initial denaturation (95 °C for 4 min), followed by 25 cycles for 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 2 min. PCR products were extracted in phenol/chloroform and precipitated using ethanol in -80 °C for one hour. The pellets were washed in 70% ethanol, dried, dissolved in H₂O and cleaved using *EcoR* I and *Xho* I, and then separately subcloned into the *EcoR* I-*Xho* I site of pGEX-4T-1 vector (Amersham Biosciences), giving ligation GST-AplVMP1 and GST-AplVMP2. Each ligation was transformed separately into XL blue competent cells (Invitrogen). Plasmid was extracted using miniprep kit (Sigma-Aldrich, USA), digested with *EcoR* I and *Xho* I for 1.5 h at 37 °C to select plasmids containing inserts of the predicted size for DNA, and further confirmed by sequencing for construction of in-frame.

2.2. Culturing methods and affinity purification

The confirmed plasmids, GST-AplVMP1 and GST-AplVMP2 were separately transformed into the *E.coli* strain BL21 star (Invitrogen) to give strain BL21/GST-AplVMP1 and BL21/GST-AplVMP2. Recombinant strain was first cultured in shaking flasks containing Luria-Bertani (LB) medium overnight. After inoculation of the overnight culture into fresh LB medium, the growth of culture cells was maintained at 37 °C and monitored turbidimetrically at 600 nm (OD₆₀₀) along the time course. Upon reaching OD₆₀₀ of 0.5, the culture was induced with a final concentration of 0.1 mM Isopropyl β-D-thiogalactoside (IPTG) for 8 h to induce of production of recombinant proteins. Samples were collected at different time points from 1 to 8 h after IPTG induction, and cells were harvested by centrifugation and resuspended in 1 × Phosphate Buffered Saline (PBS, 137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, pH 7.4). Cells were lysed on ice with a Branson Sonifier 450 (Branson, Danbury, CT) with the output control setting at 1, a duty cycle setting of constant, and 6 sonication pulses of 30 sec per pulse. The lysate was centrifuged for 10 min at 10,000 × *g*. The supernatant was affinity purified with Glutathione-Sepharose 4B beads in Econo-Colum chromatography column (BIO-RAD) on ECONO PUMP (BIO-RAD) at a flow rate of 0.03 ml/min, and then washed with 40 ml ice-cold 1 × PBS buffer at a flow rate of 0.3 ml/min. GST fusion protein was eluted from beads by 10 mM Glutathione elution buffer (Sigma-Aldrich, USA) in different fragments which were analyzed by NuPAGE 4–12% Bis-Tris Gel (Invitrogen). The concentration of mixed recombinant protein fragments was measured on DU 7400 Spectrophotometer (Beckman).

2.3. Fibrin (ogen)olytic activity

The activity of recombinant proteins was tested on fibrinogen and fibrin. Two recombinant proteins AplVMP1 and AplVMP2 as well as GST alone (negative control) and crude venom of *A. p. leucostoma* (positive control) were tested on fibrinogen. Briefly, fibrinogen (Diapharma Group, Inc, USA) solution (1.0 mg/ml in 0.1 M Tris-HCl, pH 8.5) was incubated with GST-AplVMP1, GST-AplVMP2, crude venom, and GST alone at a substrate to enzyme ratio of 33:1 (W/W) for various time intervals (10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 14 h, and 24 h) at 37 °C. Portions of incubation mixtures were withdrawn at different time points while the intact untreated fibrinogen (control) was collected at 24 h. The reactions were stopped by addition of sample denaturing buffer and analyzed by NuPAGE 4–12% Bis-Tris Gel (Invitrogen, USA).

The inhibition of the zinc-chelator EDTA (ethylenediamine tetraacetic acid), the serine protease inhibitor PMSF (phenylmethylsulphonyl fluoride) and the broad-spectrum metalloproteinase inhibitor 1,10-phenanthroline (Sigma-Aldrich, USA) on fibrinogen proteolysis by AplVMP1 and AplVMP2 was investigated by incubating each enzyme with different concentration (0, 100 and 300 μ M) of each inhibitor for 1 h at 37 °C. The two mixtures GST-AplVMP1/inhibitors and GST-AplVMP2/inhibitors were then incubated with 33 μ g of fibrinogen for 8 h at 37 °C. Samples from each reaction were analyzed by NuPAGE 4–12% Bis-Tris Gel (Invitrogen, USA).

Fibrinolytic activity was determined according to the method described by Selistre-de-Araujo et al. (2000). Briefly, a fibrin-agar gel was prepared by the addition of a final concentration 1U/ml of thrombin solution (GE healthcare) to a preheated solution of 0.9% agar in 50 mM Tris-HCl, 3 mM CaCl₂, pH 7.5, with 0.3% bovine fibrinogen (Diapharma Group, Inc, USA). The samples including GST-AplVMP1, GST-AplVMP2, GST, crude venom and GST elution buffer (GEB) were applied to the solidified gel and incubated for 24 h at 37 °C. The fibrinolytic assay was repeated at least 3 times with recombinant proteins extracted from different times and each time three plates were inoculated with different concentration of samples. One unit of fibrinolytic activity was defined as the amount of protein that produces a 1-cm halo of fibrin lysis, under the conditions described.

2.4. Autolysis assay

Recombinant proteins GST-AplVMP1 and GST-AplVMP2 were immediately incubated in room temperature from 10 min to 24 h after affinity purification. Samples were collected at different time points (10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 14 h, 24 h), stopped for autolytic reaction by adding SDS sample buffer and examined on NuPAGE 4–12% Bis-Tris Gel to check the degradation.

2.5. Blood aggregation

Blood samples, obtained by venipuncture from healthy drug-free donors, were gently mixed with 3.2% sodium citrate at the ratio of 9:1 and centrifuged at $190 \times g$ for 20 min at 25 °C. The platelet-rich plasma (PRP) was collected and the platelet count was adjusted with platelet-poor plasma (PPP) to approximately 3×10^5 /ml for aggregation assay on a Chronolog lumi-aggregometer (Chronolog Corporation, USA). Sample of 490 μ L of platelets was incubated for 4 min at 37 °C with increasing concentrations of recombinant proteins. Platelet aggregation was initiated by adding a final concentration of 10 μ M ADP or 2 μ g/ml collagen and all aggregation patterns were recorded under constant stirring in an aggregometer at 1200 rpm. The crude snake venom was used as a positive control while GST alone was used as a negative control. The experiment was repeated twice.

2.5 Hemorrhagic assay

Different doses of affinity purified recombinant proteins were immediately injected intradermally into mice. After 6 h, the animals were sacrificed and skins were removed from the belly for observation of hemorrhagic halos.

3. Results

3.1. Amino acid sequences of ApIVMP1 and ApIVMP2

Eleven full-length cDNAs encoding snake venom metalloproteinase were obtained by random sequencing of a cDNA library generated from a pair of venom glands of snake (*Agkistrodon piscivorus leucostoma*) (Jia et al., 2008). The amino acid sequences of the ten cDNAs are identical except a few amino acid changes. The comparison of amino acid sequence of these cDNA open reading frames with those in the GenBank database indicated that these cDNAs encode the same PI class of metalloproteinase. Therefore, one of the cDNA clones was selected as a representative (designated ApIVMP1, *Agkistrodon piscivorus leucostoma* venom metalloproteinase) for further investigation. The cDNA sequence of ApIVMP1 consists of 1819 nucleotides including a 84 nucleotides 5'-end untranslated region(UTR), an open reading frame of 1233 nucleotides, and a 3'-end UTR of 502 nucleotides with termination codon (Fig. 1). The full-length cDNA sequence of ApIVMP1 encodes an open reading frame of 411 amino acids that contains a cysteine switch domain PKMCGVT within a prodomain and an extended consensus catalytic site HEIGHNLMGMGHD within metalloproteinase domain (Fig. 1).

The other cDNA (designated as ApIVMP2) encoding metalloproteinase is 1966 nucleotides in length comprising 93 nucleotides of 5'-end UTR, 1371 nucleotides of open reading frame, and 502 nucleotides of 3'-end UTR with termination codon (Fig. 2). The BLAST search of deduced amino acid sequence in the database indicated that ApIVMP2 is highly identical with the precursors of the Reprolysin subfamily and belongs to the PII class of metalloproteinase containing a cysteine switch motif PKMCGVT within the prodomain, an extended consensus zinc-binding motif HEMGHNLGISHD within the metalloproteinase domain and an additional integrin receptor site KGD instead of RGD within the disintegrin domain (Fig. 2).

The protein BLAST search and CLUSTAL W multiple sequence alignments revealed that the amino acid sequence of ApIVMP1 was homologous to *Agkistrodon contortrix laticinctus* ACLPREF (U18233) (Selistre de Araujo and Ownby, 1995) and ApIVMP2 to *Agkistrodon piscivorus piscivorus* Piscivostatin(Q805F4)(Okuda and Morita, 2001) with 86% and 93% identity, respectively. Fig. 3 shows the alignment of the predicted amino acid sequence of ApIVMP1 and ApIVMP2 with other venom metalloproteinase precursors of the Reprolysin subfamily.

3.2. Production of recombinant proteins

The cDNA-coding regions for the mature ApIVMP1 and ApIVMP2 were separately amplified by PCR using clones from cDNA library as templates. The PCR products were separately subcloned into bacterial expression vector pGEX-4T-1 and transformed into *E.coli* strain XL-blue (Invitrogen). The recombinant plasmids (GST-ApIVMP1 and GST-ApIVMP2) were isolated and their sequences were further analyzed by both restriction analysis and sequencing, confirming that no substitution was introduced by PCR amplification and the inserts were in-frame with GST tag. The confirmed recombinant plasmids GST-ApIVMP1 and GST-ApIVMP2 were separately transformed into *E.coli* strain BL21 (DE3) cells, which were induced with 0.5 mM IPTG to produce the recombinant proteins. From the time-course experiments, the optimal incubation time for the maximum

production of recombinant AplVMP1 and AplVMP2 were 1 h and 3 h, respectively. However, most of recombinant proteins were in insoluble inclusion bodies (Fig. 4A, 4B), and the yields of affinity purified AplVMP1 and AplVMP2 secreted in the culture supernatant were 0.4 mg/l and 0.7 mg/l of culture medium, respectively.

To removal of GST tag from GST fusion proteins, Both GST-AplVMP1 and GST-AplVMP2 were separately incubated with thrombin protease (Amersham Biosciences) according to the instruction manual, as a result both recombinant proteins were totally degraded (data not shown) due to the auto-proteolytic processing. Therefore, the further investigated recombinant proteins were not cleaved from GST fusion proteins. The observed affinity purified GST-AplVMP1 and GST-AplVMP2 have molecular masses of 72 and 77 kDa estimated by relative mobility on SDS gels (Fig. 4), which are in accordance with the calculated molecular weights (GST for 26 kDa, AplVMP1 for 46.6 kDa and AplVMP2 for 51.1 kDa). Western blot using anti-GST antibody confirmed that the affinity purified and observed bands on SDS gel were GST fusion proteins (Fig. 4D).

3.3. Fibrin(ogen)olytic activity of AplVMP1 and AplVMP2

Since snake venom metalloproteinases are known as fibrinogenases, the effect of GST-AplVMP1, GST-AplVMP2 as well as GST alone (negative control) and crude venom (positive control) on fibrinogen was investigated. When incubated with fibrinogen, the crude venom selectively cleaved alpha and beta chain of bovine fibrinogen within 10 min of incubation at 37 °C but did not cleaved the gamma chain, even after 24 h of incubation (Fig. 5A). In the same experiment, GST-AplVMP1 started cleaving alpha chain at 8 h then the beta chain at 24 h (Fig. 5A), while AplVMP2 started cleaving alpha chain at 4 h and completely cleaved beta chain at 24 h (Fig. 5A) after incubation. The GST alone did not cleave any of the chains at any time points (Fig. 5A), which indicated that only AplVMP1 and AplVMP2 recombinant proteins in GST fusion proteins have the fibrinogenase activity.

The proteolytic activities of AplVMP1 and AplVMP2 was fully inhibited by incubation with 100 μ m of EDTA and 1,10-phenanthroline, indicating that both AplVMP1 and AplVMP2 are metal-dependent metalloproteinase. The serine protease inhibitor PMSF had no inhibitory activity against both AplVMP1 and AplVMP2, even using high concentration (300 μ m) (Fig. 5B). Both recombinant proteins underwent autolysis *in vitro*. AplVMP1 and AplVMP2 started degrading at 8 h and 2 h respectively after incubation at room temperature, indicating that AplVMP1 is more stable than AplVMP2 (data not shown). The fibrinolytic activity of GST-AplVMP1, GST-AplVMP2, GST, and GST elution buffer (GEB) and crude venom were tested on a fibrin-agars plate (Fig. 6). The fibrinolytic activities of AplVMP1, AplVMP2 and crude venom were 117 U/mg, 167 U/mg and 457 U/mg, respectively. GST alone and GEB were devoid of activity, indicating that only AplVMP1 and AplVMP2 in GST fusion proteins had the fibrinolytic activity.

3.4. Hemorrhagic activity and Platelet aggregation

To investigate whether the recombinant proteins, GST-AplVMP1 and GST-AplVMP2 have a functional role in hemorrhage, the GST-AplVMP1 and GST-AplVMP2 were injected (up to 50 μ g) subcutaneous into mice. The results indicated that both recombinant proteins were devoid of hemorrhagic activity.

A concentration-dependent inhibition of platelet aggregation (up to 30 μ g) was tested. Both recombinant proteins, GST-AplVMP1 and GST-AplVMP2 did not have any significant effect on ADP and collagen-stimulated platelet aggregation.

4. Discussion

SVMPs, one of the most destructive agents with remarkable variations in snake venom, are the primary proteinase, particularly in Crotalid and Viperid snake venoms (Bjarnason et al., 1995; Hati et al., 1999). Following snake envenomation, the SVMPs caused the characteristic pathological events resulting in local and systemic hemorrhage by action on the capillary basement membranes (Kamiguti, 2005). To advance our knowledge on SVMPs, we isolated two different classes of metalloproteinases, AplVMP1 and AplVMP2, from a snake venom gland cDNA library. The deduced amino acid sequences of AplVMP1 and AplVMP2 predict multi-domain structures. Signal peptides of AplVMP1 and AplVMP2 are comprised of 18 amino acids residues, mostly hydrophobic residues, and highly conserved with those from *C.atrox* (Bjarnason and Fox, 1994). Due to high content of hydrophobic residues, particularly in the signal sequence, some of the recombinant proteins of snake venom components expressed in *E.coli* lead to the overexpression of insoluble inclusion bodies. For example, *Agkistrodon contortrix laticinctus* recombinant ACLF (P-III type metalloproteinase) was overexpressed in *E.coli* and rACLF directed to inclusion bodies (Ramos et al., 2003). MT-d (P-II type metalloproteinase) isolated from *Agkistrodon halys brevicaudus* was also in insoluble fragment when expressed in *E.coli* (Jeon and Kim, 1999). We tested other venom recombinant proteins including disintegrins, lectins and phospholipases in both prokaryotic (*E.coli*) and eukaryotic expression (Sf9 insect cell) systems. The expressed recombinant proteins were in insoluble inclusion bodies and difficult to purify by normal affinity purification system, but they were soluble after cleaving the signal peptide (data not shown). AplVMP1 and AplVMP2 were partially soluble although most of the recombinant proteins were in insoluble inclusion bodies (Fig. 4A and 4B). We adjusted *E.coli* incubation temperature from 37 °C to 15 °C but there was no change in soluble recombinant protein yield. Therefore, the only strategy to obtain enough amounts of recombinant proteins is to increase the amount of bacterial culture harboring the recombinant plasmid. We increased the amount of bacterial culture up to 4 liters and obtained 1.6 mg and 2.8 mg recombinant proteins for AplVMP1 and AplVMP2, respectively. In addition, the affinity purified AplVMP1 and AplVMP2 in Fig. 4 showed at least two major bands, the possible explanations for these bands are: (1) Due to the hemorrhagic toxin of metalloproteinase, *E.coli* enzymes partially digested metalloproteinase in the GST fusion proteins. Therefore there are at least two bands (GST-Aplomb and GST alone), which were recognized by anti-GST antibody (Fig. 4D). (2) Due to auto-proteolytic processing of metalloproteinase, both AplVMP1 and AplVMP2 might be partially degraded. Thus, there are two major bands and several weak bands.

The pro-domain of AplVMP1 is comprised of 169 amino acid residues while that of AplVMP2 is composed of 144 amino acid residues with 26 amino acid residues truncated in comparison to amino acid sequence of AppVMP from *Agkistrodon piscivorus piscivorus* (Okuda et al., 2001) (Fig. 3). In the pro-domains of AplVMP1 and AplVMP2, the consensus sequence PKMCGVT is homologous to the cysteine switch sequence of matrix metalloproteinases (MMPs) and other reprolysins. The pro-domain keeps metalloproteinases as latent enzymes which must be proteolytically activated, usually by hydrolysis of the pro-domain (Stocker et al., 1995). Therefore, the pro-domains in AplVMP1 and AplVMP2 may play a similar role during their biosynthesis, and may involve in the mechanism of pro-enzyme latency as it is in the case of the MMPs. They may also modulate the enzymatic activity through interactions with the catalytic domain.

Both metalloproteinase domains of AplVMP1 and AplVMP2 are comprised of 224 amino acid residues with the characteristic consensus sequence of the zinc-binding motif HEXGHXXGXXHD and the structural Met-turn CIM. The zinc-binding motif is involved in catalysis in their metalloproteinase domain (Stocker et al., 1995) and hence has protease

activity. The zinc-binding motifs of AplVMP1 and AplVMP2 consist of three His residues and a water molecule that tetrahedrally coordinates the zinc and the Glu residue acts as a catalytic base. An Asp residue is located at the end of the zinc-binding motif. AplVMP1 consists of a pro-domain and a metalloproteinase domain, whereas AplVMP2 comprises an additional disintegrin domain that comprised 71 amino acids with 10 Cys residues. We previously isolated a cDNA clone encoding a disintegrin with an RGD motif from the same cDNA library in which the AplVMP1 and AplVMP2 were obtained. The cDNA sequence comparison of the disintegrin with the disintegrin domain of AplVMP2 showed 42% identity, their signal sequences are 89% identical and the first 13 amino acid residues of pro-domain are identical (data not shown). Disintegrins are cysteine-rich proteins, which usually contain an Arg-Gly-Asp (R-G-D) or a Lys-Gly-Asp (K-G-D) structural motif recognized by platelet integrin $\alpha_{IIb} \beta_3$ and may involved in cell-cell interactions. The conserved cysteines are involved in disulfide bond formation and are important for maintaining molecular structure and biochemical functions (Calvete et al., 2003, 2005). The disintegrin domain of AplVMP2 contains KGD motif instead of RGD and 10 cysteines (Fig. 2), and might possess the similar functions of maintaining molecular structure, but remains to be determined whether the KGD motif in AplVMP2 can bind platelet integrins or cysteine residues involved in disulfide bond formation.

SVMPs are frequently responsible for the degradation of matrix proteins, resulting in the disruption of endothelial cell integrity in blood capillary vessel walls and subsequent hemorrhage that is exacerbated by disturbances in platelet function (Moreira et al., 1994; Bjarnason and Fox, 1994; Kamiguti et al., 2003). SVMPs also effectively digest large proteins involved in haemostasis, such as fibrinogen. Therefore, the capacity of affinity purified recombinant proteins, AplVMP1 and AplVMP2, to metabolize human fibrinogen was evaluated. Both recombinant proteins were more effective on the alpha chain of fibrinogen than on the beta chain, without cleavage of the gamma chain. The effect of AplVMP1 and AplVMP2 to hydrolyze the substrate fibrinogen is appreciably different. AplVMP1 started cleaving alpha chain at 8 h and then beta chain at 24 h while AplVMP2 started cleaving alpha chain at 4 h and then completely cleaved beta chain at 24 h after incubation at 37 °C, but both recombinant proteins failed to cleave gamma chain at any time point (Fig. 5A). To exclude GST involved in the activity assay, we tested GST recombinant protein alone under the same experiment conditions, found that GST alone was devoid of fibrinogenolytic activity (Fig. 5A). Crude venom as a positive control cleaved both alpha and beta chains within 10 min and started digesting gamma chain at 24 h (Fig. 5A). These results demonstrated that only AplVMP1 and AplVMP2 have the fibrinogenolytic activity. Similarly, Howes et al. (2005) confirmed that EoVMP2 (P-III metalloproteinase) selectively cleaved α -chain and EoVMP3 (P-III metalloproteinase) cleaved α and β -chain, whereas EoVMP1 (P-I metalloproteinase) was devoid of proteolytic activity.

Fibrinogen functions as a major ligand for the platelet $\alpha_{IIb} \beta_3$ integrin, it was thought that its degradation could be responsible for the inhibition of platelet aggregation (Kamiguti, 2005). However, both AplVMP1 and AplVMP2 selectively digested alpha and beta chain but did not degrade gamma chain which contains the more important platelet-binding site (Kamiguti, 2005). Therefore, the fibrinogen digestion alone is not sufficient to explain the hemorrhagic activity of both AplVMP1 and AplVMP2. We further verified that both GST-AplVMP1 and GST-AplVMP2 are devoid of hemorrhagic activity when injected (up to 50 μ g) subcutaneous into mice.

The fibrinogenolytic activity of AplVMP1 and AplVMP2 was completely inhibited by a final concentration of 100 μ M of the zinc-chelator EDTA and metalloproteinase inhibitor 1,10-phenanthroline, and thus AplVMP1 and AplVMP2 did not cleave any chains of fibrinogen, even incubated with 300 μ M of inhibitors for 8 h at 37 °C, which indicated that

both enzymes are metal-dependant proteinase and the activity of AplVMP2 is slightly stronger than that of AplVMP1 (Fig. 5B). In addition, the serine protease inhibitor PMSF did not show any effect on fibrinogen, even using high concentration of 300 μm (Fig. 5B).

Both recombinant proteins underwent autolysis *in vitro*. AplVMP1 started degrading from 8 h whereas AplVMP2 from 2 h after incubated at room temperature (data now shown), indicating that AplVMP1 is more stable than AplVMP2. The autolysis is the main reason why we were not able to remove GST tag from fusion proteins. The addition of inhibitor EDTA to GST-fusion proteins can inhibit autolysis but it affects the subsequent activity detection.

Our previous research results (Jia et al., 2008) also showed that SVMPs underwent proteolytic processing before their presence in the venom, because the transcripts statistic data show that metalloproteinases make up 2.8%, the second largest component in *A.p. leucostoma* transcripts. Consequently, a strong protein band with molecular mass more than 49 kDa should appear in *A.p.leucostoma* venom SDS gel, but amino acid sequence of protein bands of *A.p.leucostoma* venom showed that there is no metalloproteinase. Similarly, Jeon et al. (1999) and Ramos et al. (2003) also demonstrated the autolysis of recombinant SVMPs from *Agkistrodon halys brevicaudus* and *Agkistrodon contortrix laticinctus*.

In summary, we identified two low-molecular weight metalloproteinase, AplVMP1 and AplVMP2, from a snake (*A.p.leucostoma*) venom gland cDNA library. On the basis of amino acid sequences, AplVMP1 and AplVMP2 belong to PI and PII class enzymes, respectively. Both recombinant proteins, AplVMP1 and AplVMP2 with fibrin(ogeno)lytic and autolytic activities, were successfully expressed in the *E.coli* expression system and affinity purified. Our research results also suggest that AplVMP1 and AplVMP2, by virtue of their fibrinogenolytic feature, may be used as potential clinical agents to treat occlusive thrombi, or as an important tool to better understand the action mechanism of similar snake venom toxins.

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Abbreviations

AplVMP	<i>Agkistrodon piscivorus leucostoma</i> snake venom metalloproteinase
cDNA	complementary DNA
GST	glutathione <i>S</i> -transferase
ADAMs	A Disintegrin And Metalloproteinases
PCR	Polymerase chain reaction
PRP	platelet-rich plasma
PPP	platelet-poor plasma
ADP	Adenosine diphosphate
EDTA	ethylenediaminetetraacetic acid
PMSF	phenylmethylsulphonyl fluoride
MMP	matrix metalloproteinase

References

- Assakura MT, Silva CA, Mentele R, Camargo ACM, Serrano SMT. Molecular cloning and expression of structural domains of bothropasin, a P-III metalloproteinase from the venom of *Bothrops jararaca*. *Toxicon*. 2003; 41:217–227. [PubMed: 12565741]
- Bello CA, Hermogenes ALN, Magalhaes A, Veiga SS, Gremski LH, Richardson M, Sanchez EF. Isolation and biochemical characterization of a fibrinolytic proteinase from *Bothrops leucurus* (white-tailed jararaca) snake venom. *Biochimie*. 2006; 88:189–200. [PubMed: 16139412]
- Bernardes CP, Santos-Filho NA, Costa TR, Gomes MSR, Torres FS, Costa J, Borges MH, Richardson M, dos Santos DM, de Castro Pimenta AM, Homsí-brandeburgo MI, Soares AM, de Oliveira F. Isolation and structural characterization of a new fibrinogenolytic metalloproteinase from *Bothrops moojeni* snake venom. *Toxicon*. 2008; 51:574–584. [PubMed: 18187176]
- Birkedal-Hansen H. Proteolytic remodeling of extracellular matrix. *Curr Opin Cell Biol*. 1995; 7:728–735. [PubMed: 8573349]
- Bjarnason JB, Fox JW. Hemorrhagic metalloproteinases from snake venom. *Pharmacol Ther*. 1994; 62:325–372. [PubMed: 7972338]
- Bjarnason, JB.; Fox, JW. In handbook of proteolytic enzymes. Baqrrrett, AJ.; Rawlings, ND.; Woessner, JF., editors. Academic press; San Diego: 1998. p. 1247-1254.
- Calvete JJ, Moreno-Murciano MP, Theakston RD, Kisiel DG, Marcinkiewicz C. Snake venom disintegrins: novel dimeric disintegrins and structural diversification by disulphide bond engineering. *Biochem J*. 2003; 372:725–734. [PubMed: 12667142]
- Calvete JJ, Marcinkiewicz C, Monleon D, Esteve V, Celda B, Juarez P, Sanz L. Snake venom disintegrins: evolution of structure and function. *Toxicon*. 2005; 15:1063–1074. [PubMed: 15922775]
- Chen HS, Chen JM, Lin CW, Khoo KH, Tsai IH. New insights into the functions and *N*-glycan structures of factor X activator from Russell's viper venom. *FEBS Journal*. 2008; 275:3944–3958. [PubMed: 18616470]
- Fernandez JH, Silva CA, Assakura MT, Camargo ACM, Serrano SMT. Molecular cloning, functional expression, and molecular modeling of bothrostatin, a new highly active disintegrin from *Bothrops jararaca* venom. *Biochemical and Biophysical Research Communications*. 2005; 329:457–464. [PubMed: 15737609]
- Fox JW, Serrano SM. Structural consideration of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. *Toxicon*. 2005; 45:969–985. [PubMed: 15922769]
- Gay CC, Leiva LC, Marunak S, Teibler P, Acosta de Perez O. Proteolytic, edematogenic and myotoxic activities of a hemorrhagic metalloproteinase isolated from *Bothrops alternates* venom. *Toxicon*. 2005; 46:546–554. [PubMed: 16137735]
- Gowda DC, Jackson CM, Hensley P, Davidson EA. Factor X-activating glycoprotein of Russell's viper venom. Polypeptide composition and characterization of the carbohydrate moieties. *J Biol Chem*. 1994; 269:10644–10650. [PubMed: 8144654]
- Gutiérrez JM, Rucavado A. Snake venom metalloproteinases: their role in the pathogenesis of local tissue damage. *Biochimie*. 2000; 82:841–850. [PubMed: 11086214]
- Hati R, Mitra P, Sarker S, Bhattacharyya KK. Snake venom hemorrhagins. *Crit Rev Toxicol*. 1999; 29:1–19. [PubMed: 10066158]
- Hite LA, Shannon JD, Bjarnason JB, Fox JW. Sequence of a cDNA clone encoding the zinc metalloproteinase hemorrhagic toxin e from *Crotalus atrox*: evidence for signal zymogen, and disintegrin-like structure. *Biochemistry*. 1992; 31:6203–6211. [PubMed: 1378300]
- Hite LA, Jia LG, Bjarnason JB, Fox JW. cDNA sequences for four snake venom metalloproteinases: structure, classification, and their relationship to mammalian reproductive proteins. *Arch Biochem Biophys*. 1994; 308:182–191. [PubMed: 8311451]
- Hooper NM. Families of zinc metalloproteinases. *FEBS Letters*. 1994; 354:1–6. [PubMed: 7957888]
- Howes JM, Kamiguti AS, Theakston RDG, Wilkinson MC, Laing GD. Effects of three novel metalloproteinases from the venom of the West African saw-scaled viper, *Echis ocellatus* on blood

- coagulation and platelets. *Biochimica et Biophysica Acta*. 2005; 1724:194–202. [PubMed: 15863354]
- Jeon OH, Kim DS. Molecular cloning and functional characterization of a snake venom metalloproteinase. *Eur J Biochem*. 1999; 263:526–533. [PubMed: 10406963]
- Jia LG, Wang XM, Shannon DJ, Bjarnason JB, Fox JW. Function of disintegrin-like/cysteine-rich domains of Atrolysin A. inhibition of platelet aggregation by recombinant protein and peptide antagonists. *J Biol Chem*. 1997; 272:13094–13102. [PubMed: 9148922]
- Jia Y, Cantu BA, Sánchez EE, Pérez JC. Complementary DNA sequencing and identification of mRNAs from the venomous gland of *Agkistrodon piscivorus leucostoma*. *Toxicon*. 2008; 51:1457–1466. [PubMed: 18502463]
- Kamiguti AS, Gallager P, Marcinkiewicz C, Theakston RGD, Zuzel M, Fox JW. Identification of sites in the cysteine-rich domain of the class P-III snake venom metalloproteinase responsible for the inhibition of platelet function. *FEBS Lett*. 2003; 549:129–134. [PubMed: 12914938]
- Kamiguti AS. Platelets as targets of snake venom metalloproteinases. *Toxicon*. 2005; 45:1041–1049. [PubMed: 15922773]
- Leonardi A, Fox JW, Trampuš-Bakija A, Križaj I. Two coagulation factor X activators from viper a ammodytes venom with potential to treat patients with dysfunctional factors IXa or VIIa. *Toxico*. 2008; 52:628–637.
- Marcussi S, Bernardes CP, Santos-filho NA, Mazzi MV, Oliveira CZ, Izidoro LFM, Fuly AL, Magro AM, Braz ASK, Fontes MRM, Giglio JR, Soares AM. Molecular and functional characterization of a new non-hemorrhagic metalloproteinase from *Bothrops jararacussu* snake venom with antiplatelet activity. *Toxicon*. 2007; 28:2328–2339.
- Mazzi MV, Marcussi S, Carlos GB, Stabeli RG, Franco JJ, Tieli FK, Cintra ACO, Franca SC, Soares AM, Sampaio SV. A new hemorrhagic metalloproteinase from *Bothrops jararacussu* snake venom: isolation and biochemical characterization. *Toxicon*. 2004; 44:215–223. [PubMed: 15246772]
- Moreira L, Borkow G, Ovadia M, Gutiérrez JM. Pathological changes induced by BaH1, a hemorrhagic proteinase isolated from *Bothrops asper* (terciopelo) snake venom, on mouse capillary blood vessels. *Toxicon*. 1994; 32:977–987.
- Mori N, Nikai T, Sugihara H. Purification of a proteinase (Ac₅-proteinase) and characterization of hemorrhagic toxins from the venom of the hundred-pace snake (*Agkistrodon acutus*). *Toxicon*. 1984; 22:451–461. [PubMed: 6433514]
- Okuda D, Morita T. Purification and characterization of a new RGD/KGD-containing dimeric disintegrin, piscivostatin, from the venom of *Agkistrodon piscivorus piscivorus*: the unique effect of piscivostatin on platelet aggregation. *J Biochem*. 2001; 130:407–415. [PubMed: 11530017]
- Ramos OHP, Carmona AK, Selistre-de-Araujo HS. Expression, refolding, and in vitro activation of a recombinant snake venom pro-metalloproteinase. *Protein Expression and Purification*. 2003; 28:34–41. [PubMed: 12651104]
- Rodrigues FG, Petretski JH, Kanashiro MM, Lemos L, da Silva WD, Kipnis TL. The complement system is involved in acute inflammation but not in the hemorrhage produced by a *Bothrops atrox* snake venom low molecular mass proteinase. *Mol Immunol*. 2004; 40:1149–1156. [PubMed: 15104120]
- Selistre-de-Araujo HS, Ownby CL. Molecular cloning and sequence analysis of cDNAs for metalloproteinases from Broad-banded copperhead *Agkistrodon contortrix laticinctus*. *Archives of Biochemistry and Biophysics*. 1995; 320:141–148. [PubMed: 7793974]
- Selistre-de-Araujo HS, Dulce HF, Ownby CL. Analysis of a cDNA sequence encoding a novel member of the snake venom metalloproteinases, disintegrin-like, cysteine-rich (MDC) protein family from *Agkistrodon contortrix laticinctus*. *Biochim Biophys Acta*. 1997; 1342:109–115. [PubMed: 9392519]
- Selistre-de-Araujo HS, de Souza EL, Beltramini LM, Ownby CL, Souza DHF. Expression, refolding, and activity of a recombinant nonhemorrhagic snake venom metalloproteinase. *Protein Expression and Purification*. 2000; 19:41–47. [PubMed: 10833388]
- Siigur E, Tonismagi K, Trummal K, Samel M, Vija H, Subbi J, Siigur J. Factor X activator from *Vipera lebetina* snake venom, molecular characterization and substrate specificity. *Biochim Biophys Acta*. 2001; 1568:90–98. [PubMed: 11731090]

- Siigur E, Aaspõllu A, Trummal K, Tonismagi K, Tammiste I, Kalkkinen N, Siigur J. Factor X activator from *Vipera lebetina* venom is synthesized from different genes. *Biochem Biophys Acta*. 2004; 1702:41–51. [PubMed: 15450849]
- Singhamatr P, Rojnuckarin P. Molecular cloning of albolatin, a novel snake venom metalloproteinase from green pit viper (*Trimeresurus albolabris*), and expression of its disintegrin domain. *Toxicon*. 2007; 50:1192–1200. [PubMed: 17870140]
- Stocker W, Grams F, Baumann U, Reinemer P, Gomis-Ruth FX, McKay DB, Bode W. The metzincins topological and sequential relations between astacins, adamalysins, serralsins, and matrixins (collagenases) define a super family of zinc-peptidases. *Protein science*. 1995; 4:823–840. [PubMed: 7663339]
- Stroka A, Donato JL, Bon C, Hyslop S, Lôbo de Araújo A. Purification and characterization of a hemorrhagic metalloproteinase from *Bothrops lanceolatus*(Fer-de-lance) snake venom. *Toxicon*. 2005; 45:411–420. [PubMed: 15733562]
- Swenson S, Costa F, Minea R, Sherwin RP, Ernst W, Fujii G, Yang D, Markland FS Jr. Intravenous liposomal delivery of the snake venom disintegrin contortrostatin limits breast cancer progression. *Mol Cancer Ther*. 2004; 3:499–511.
- Takeda S, Igarashi T, Mori H. Crystal structure of RVV-X: An example of evolutionary gain of specificity by ADAM proteinases. *FEBS Letters*. 2007; 581:5859–5864. [PubMed: 18060879]
- Toombs CF. Alfimeprase: pharmacology of a novel fibrinolytic metalloproteinase for thrombolysis. *Haemostasis*. 2001; 31:141–147. [PubMed: 11910179]
- Watanabe L, Shannon JD, Valente RH, Rucavado A, Alape-Girón A, Kamiguti AS, Theakston RDG, Fox JW, Gutiérrez JM, Arni RK. Amino acid sequence and crystal structure of BaP1, a metalloproteinase from *Bothrops asper* snake venom that exerts multiple tissue-damaging activities. *Protein science*. 2003; 12:2273–2281. [PubMed: 14500885]

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acggccgggggtcacagaggaagctcaggttggttgaaagaaggaagagattgcctgtctccagccaaatcca
gctccaaaaatgatccagggtctcttggtgactatattgcttagcagcttttctctatcaaggagctctataatc
M I Q V L L V T I C L A A F P Y Q G S S I
Signal sequence
ctggaatctgggaacgttaagtattgaaatagtgtatccacgaaaagtcactccagtgccagaggagcagtt
L E S G N V N D Y E I V Y P R K V T P V P R G A V
cagccaaagtatgaagatgccatgcaatgaattgaaagtgaatggagagccagtggtcctccactggaaaaa
Q P K Y E D A M Q Y E L K V N G E P V V L H L E K
aataaaggactttttcagaagattacagcgagactcattattccctgatggcagagaaattacaacatacccc
N K G L F S E D Y S E T H Y S P D G R E I T T Y P
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L V E D H C Y Y H G R I E N D A D S T A S I S T C
Pro-domain
aacggtttgaaaggacatttcaagcttcaaggggagatgtacctattgaaccgttggagccttccgacagtgaa
N G L K G H F K L Q G E M Y L I E P L E L S D S E
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A H A V Y K Y E N V E K E D E A P K M C G V T Q N
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W E S Y E P T K K A F Q L N L T P E Q Q G F P Q R
tacgttgagcttgatcataatgcagatcacagaatgtacatgaaatatacaaacgtgattcaataagatacacia
Y V E L V I I A D H R M Y M K Y K R D S N K I T Q
tgggtacatcaaatggtcaacactataaatgagatttacagacccttgatattcaattcgcactggttgcccta
W V H Q M V N T I N E I Y R P L N I Q F A L V G L
gaaatttggtccaaccaagatttgattaccgtgacgtcagatcagatgatactttgatatttgcaaacctgg
E I W S N Q D L I T V T S V S D D T L I S F A N W
Metalloproteinase domain
agagagacagctcttgctaaaggcgaagtcacatgataatgcccaattactcagccattgtcttcgatgaagga
R E T V L L R R K S H D N A Q L L T A I V F D E G
attataggaagagctcccctagccggcatgtgagaccgaaatcgttctgtaggaactgtccaggatcatagcaaa
I I G R A P L A G M C D P N R S V G T V Q D H S K
ataaatttcgggttgcaattataatggccatgagataggtcataatctgggcatgggtcagcagataatcc
I N F R V A I I M A H E I G H N L G M G H D D N S
tgtacttgcggtgatactcagcattatgttgcaccggttaagtaacaaccttcaaacattcagcagattgt
C T C G G Y S C I M L P R L S K Q P S K L F S D C
agtaagaagactatctgacgtttcttaagttaaaaaccacaatgcattctcaataaaccttgagaacagat
S K K D Y L T F L K V K N P Q C I L N K P L R T D
actgtttcaactccagtttctggaatgaacttttgaggcgtgagaagaatgtgactgtggcttctcctgacgtt
T V S T P V S G N E L L E A -
tgcaccaacagggcagtggttgatgtgactacagcctaataataaacctctggcttcttccagatttgatcttg
agatcttcttccagaaggttggcttccctgtagtccaagagaccatctgcctgcactactagtaataatca
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aaaaaaaaaaaaaaaaaaaaa

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Fig. 1. Complete cDNA and deduced amino acid sequences of the AplVMP1 with the translated open reading frame from the start codon ATG to the termination codon TGA (both in italic). The cDNA sequence including coding region, 5'- and 3'-end untranslated regions are shown in lowercase letters. The deduced amino acid sequence is shown in uppercase. The cysteine-switch motif is single underlined, while zinc-binding motif and Met-turn motifs are double underlined and boxed respectively. The poly (A) addition signal is shaded and each domain is indicated by arrows.

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ttccttgcttctcatagtcacacagaggaagagctcaggttggcttgaagaaggaagagattgctgcttctccag
ccgaatccagcctccaaaatgatccaagttctcttggtagctctatgcttagcagcttttcccttatcaagggaac
      M I Q V L L V T L C L A A F P Y Q G N
      Signal sequence
tctataatcctggaatctgggaatgttaatgattatgaatttaaagtgaatggagagccagtggtccttcacctg
S I T L E S G N V N D Y E F K V N G E P V V L H L
gaaaaaaataaaggactttttcaaaagattacacgagactcattattcctctgtagggcagaaaaattacaaca
E K N K G L F S K D Y S E T H Y S S D G R K I T T
aacctcctgggtgagatcattgtattatcatggacgcacccagaatgatgctgactcaactgcaagcatcgt
N P P V E D H C Y Y H G R I Q N D A D S T A S I S
      Pro-domain
gcatgcaacgggttgaaggacatttcaagcttcaaggggagacgtacctattgaaccttgaagctttccgac
A C N G L K G H F K L Q G E T Y L I E P L K L S D
agtgaagcccatgagcttacaatatgaaaacgtagaaaaagaagatgagcccccataatgtgtgggtaacc
S E A H A V Y K Y E N V E K E D E A P K M C G V T
cagactaatggaaatcagatagggccatcaaaaaggcctctcagttaaatcttactcctgaacaacaagattc
Q T N W K S D R P I K K A S Q L N L T P E Q Q R F
ccccaaagatacattgagcttgtttagttgagatcacagaatgttccagaaatacaacggcaattttaaact
P Q R Y I E L V V V A D H R M F T K Y N G N L N T
attagaatatgggtacatgaacttgcacactatgaatgtgttttacagaccttgaatattcatgtctcactg
I R I W V H E L V N T M N V F Y R P L N I H V S L
actgacctagaagttggtcagaccaagatttgatcaacgtgcagccagcagcagctgatacttgaagcatt
T D L E V W S D Q D L I N V Q P A A A D T L E A F
      Metalloprotease domain
ggagactggagagagacagctcttgctgaatcgcataagtcagataatgctcagttactcacggccattgagctt
G D W R E T V L L N R I S H D N A Q L L T A I E L
gatggagaaactataggttggctaacaggggaccatgtgacccgaagctttctacaggaattgttcaggat
D G E T I G L A N R G T M C D P K L S T G I V Q D
catagtgcaataaactttgggttcagttacaatggccatgagatgggtcataatctgggcattagtcacgat
H S A I N L W V A V T M A H E M G H N L G I S H D
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G N Q C H C D A N S C I M S E E L R Q Q L S F E F
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S D C S Q N Q Y Q T F L T D H N P Q C M L N E P L
agaacagatattgttcaactccagtttctggaatgaactttgggagacgggagaagaagtgactttgacgct
R T D I V S T P V S G N E L W E T G E E S D F D A
cctgcaaatccgtgctgcatgtaaacctgtaaacctgagaccaggggacagtgctgcagaaggactgtgtgtg
P A N P C C D A E T C K L R P G A Q C A E G L C C
      Disintegrin-like domain
gaccagtgcaatttatgaagaaggaacagctatgccacagagcaaaggggtgatggcctggatgattactgcaat
D Q C K F M K E G T V C H R A K...G...D G L D D Y C N
ggcatatctgctggctgtcccagaaatcccttccatgctaaccaacaatggagatggaatgggttgcagcaaca
G I S A G C P R N P F H A -
Ggcagtggttgatgtaatacagcctaataatcaacctctggctttttcagatttgatcagggatcctttt
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aaaaaaaaaaaaaaaaaaaa

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Fig. 2. cDNA sequence of ApIVMP2 encoding metalloproteinase with the translated open reading frame from the start codon ATG to the termination codon TAA (both in italic). The cysteine-switch motif is single underlined, while zinc-binding motif and Met-turn motifs are double underlined and boxed respectively. ApIVMP2 has a KGD motif (dotted line) within the disintegrin-like domain. The poly (A) addition signal is shaded and each domain is indicated by arrows.

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AppVMP  MIQVLLVTLCLAAFPYQGNSIIILESGNVNDYEVLYPQKVTALPKGAVQPKYEDTMQYEFKVNGEPVVLI 69
AplVMP2  MIQVLLVTLCLAAFPYQGNSIIILESGNVN-----DYEYFKVNGEPVVLI 43
AclVMP   MIQVLLVTLCLAAFPYQGSSIIILESGNVNDYEVVYPRKVTVPVPRGAVQPKYEDAMQYEFKVNGEPVVLI 69
AplVMP1  MIQVLLVTLCLAAFPYQGSSIIILESGNVNDYEVVYPRKVTVPVPRGAVQPKYEDAMQYELKVNGEPVVLI 69

AppVMP  LEKNKGLFSKDYSETHYSSDGRKITTNPPVEDHCYHGRIQNDADSTASISACNGLKGHFKLQGETYLI 138
AplVMP2  LEKNKGLFSKDYSETHYSSDGRKITTNPPVEDHCYHGRIQNDADSTASISACNGLKGHFKLQGETYLI 112
AclVMP   LEKNKGLFSEDYSETHYSPDGREITTYPLVEDHCYHGRRIENDADSTASISACNGLKGHFKLQGEYLI 138
AplVMP1  LEKNKGLFSEDYSETHYSPDGREITTYPLVEDHCYHGRRIENDADSTASISTCNGLKGHFKLQGEYLI 138

AppVMP  EPLKLSDEAHAVYKYENVEKEDEAPKMGVQTQNWKSDRPIKASQLNLTPEQQRFPQRYIELVVAD 207
AplVMP2  EPLKLSDEAHAVYKYENVEKEDEAPKMGVQTQNWKSDRPIKASQLNLTPEQQRFPQRYIELVVAD 181
AclVMP   EPLELSDSEAHAVYKYENVEKEDEAPKMGVQTQ--NWESYEPKKAQFQLNLTPEQGFQRYVELVIVAD 206
AplVMP1  EPLELSDSEAHAVYKYENVEKEDEAPKMGVQTQ--NWESYEPTKKAQFQLNLTPEQGFQRYVELVIVAD 206

AppVMP  HRMFTKYNGNLNTRIRIWHVELVNTMNVFYRPLNIHVSLTDLEVWSDQDLINVPAAADTLEAFGDWRET 276
AplVMP2  HRMFTKYNGNLNTRIRIWHVELVNTMNVFYRPLNIHVSLTDLEVWSDQDLINVPAAADTLEAFGDWRET 250
AclVMP   HRMNTKYNGSDKIRQNVHIVNTINEIYRPLNIRFALVGLIWSNQDLITVTSVSHDTLASFGNWRET 275
AplVMP1  HRMYMKYKRDNSNKITQVHVQMVNTINEIYRPLNIQFALVGLIWSNQDLITVTSVSDDTLISFANWRET 275

AppVMP  VLLNRI SHDNAQLLTAIELDGETIGLANRGTMCDPKLSGTGIVQDHSAINLWVAVTMAHEMGHNLGISHD 345
AplVMP2  VLLNRI SHDNAQLLTAIELDGETIGLANRGTMCDPKLSGTGIVQDHSAINLWVAVTMAHEMGHNLGISHD 319
AclVMP   DLLRRQRHDNAQLLTAIDFDGDTVGLAYVGGMCQLKHSSTGVIQDHSAINLLVALTMAHELGHNLGMNHD 344
AplVMP1  VLLRRKSHDNAQLLTAIVFDEGIIGRAPLAGMCDPNRSVGTVDHSGINFRVAIIMAHEIGHNLGMGHD 344

AppVMP  GNQCHCDANSCIMSEELRQQLSFEFSDCSQNQYQTFLDHNPQCMLNEPLRTDIVSTPVSGNELWETGE 414
AplVMP2  GNQCHCDANSCIMSEELRQQLSFEFSDCSQNQYQTFLDHNPQCMLNEPLRTDIVSTPVSGNELWETGE 388
AclVMP   GNQCHCGANSCVMPVSLDQPSKLFSDCSKKDYQTFLPVNNPQCILNKPLRTDTASTPVSGNELLEA-- 411
AplVMP1  DNSCTCGGYSXIMPLPRLSKQPSKLFSDCSKKDYLTFLKVNPNQCILNKPLRTDTVSTPVSGNELLEA-- 411

AppVMP  ESDFDAPANPCDDAATCKLTPGSQCAEGLCCDQCKFMKEGTVCHRAKGDDLDYDNGISAGCPRNPFHA 483
AplVMP2  ESDFDAPANPCDDAETCKLRPGAQCAEGLCCDQCKFMKEGTVCHRAKGDDLDYDNGISAGCPRNPFHA 457
AclVMP   -----
AplVMP1  -----
    
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Fig. 3. Comparison of deduced amino acid sequences of AplVMP1 and AplVMP2 with two other members of the Reprolysin family. The amino acid sequences are numbered from the N-terminal amino acid of the signal peptide. AplVMP2 has a 93% identity with AppVMP isolated from *Agkistrodon piscivorus piscivorus* with accession number Q805F4 (Okuda et al., 2002). AplVMP1 is 85% identical with AclVMP isolated from *Agkistrodon contortrix laticinctus* with accession number of U18233 (Selistre et al, 1995). This alignment was generated with CLUSTALW multiple sequence alignment programs and BOX shade, followed by manually. Amino acid residues identical with and similar to those of other members are shown with black shading and grey shading respectively.

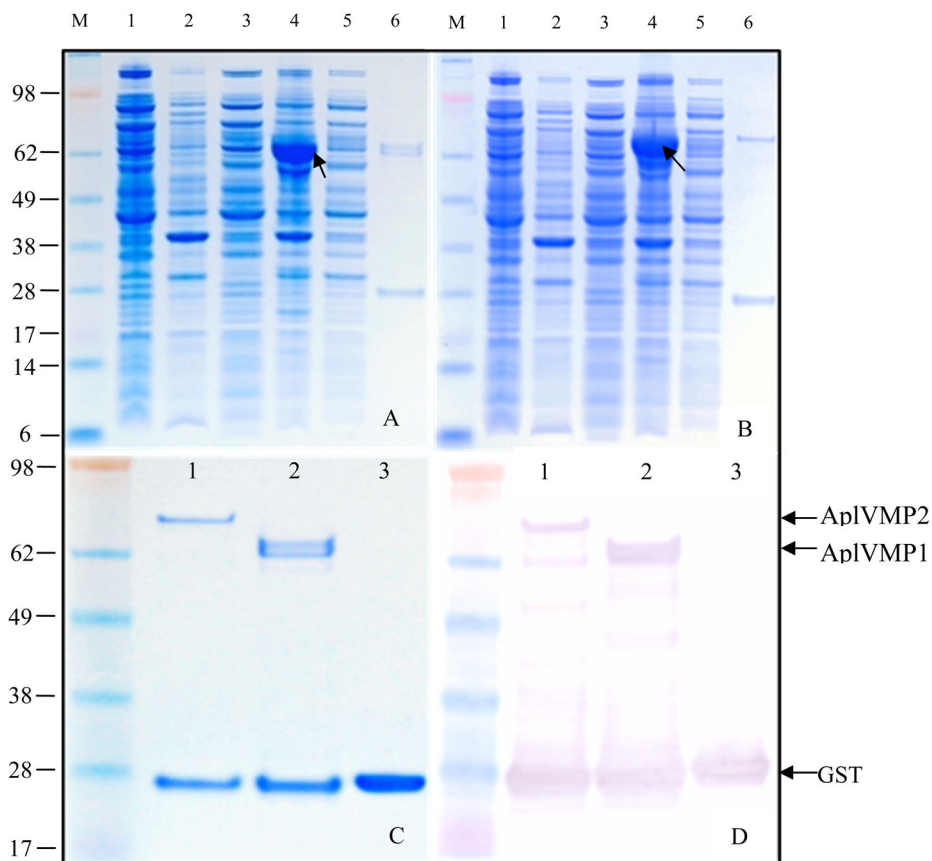


Fig. 4. Affinity purification of AplVMP1 and AplVMP2 showing on 4–12% SDS-PAGE gels under reducing condition and coomassie blue stained. Upper panel: A and B represent *E.coli* cells harboring AplVMP1 and AplVMP2, respectively. Lanes: 1, *E.coli* cell pellet without harboring recombinant plasmids; 2, Cell pellet before IPTG induction; 3, Supernatant before IPTG induction; 4, Cell pellet after induction; 5, Supernatant after induction; 6, GST-AplVMP1 and GST-AplVMP2 after affinity purification from 5 respectively. C, Affinity purified AplVMP1, AplVMP2 and GST alone on coomassie stained gel. 1, AplVMP2; 2, AplVMP1; 3, GST alone. D, Western blot using anti-GST antibody to confirm the affinity purified GST fusion proteins. Arrows in A and B show the induced recombinant proteins in inclusion bodies. M shows the standard molecular weight (kDa).

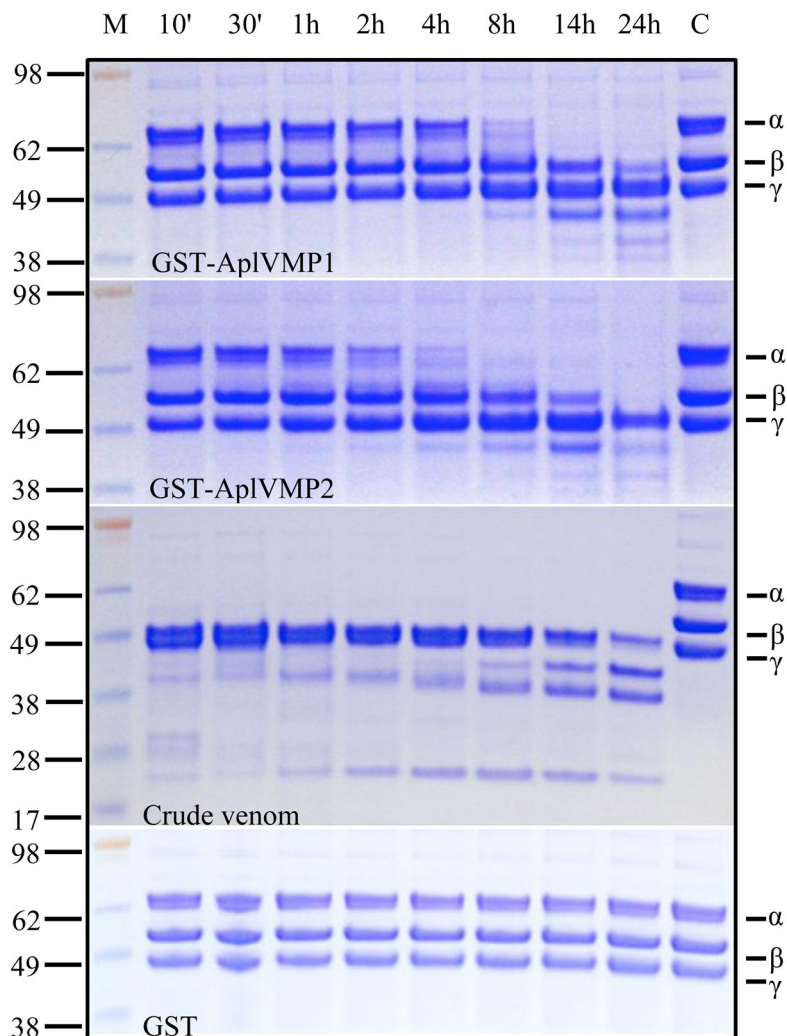


Fig. 5. Proteolysis of bovine fibrinogen by different enzymes. Fibrinogen was incubated with different enzymes at a substrate to enzyme ratio of 33:1 (W/W) for a total time of 24 hours at 37 °C. Aliquots from the incubation mixture were removed at indicated time. Migration pattern of the fragments formed during reaction were analyzed on 4–12% SDS-PAGE under reduced condition and coomassie blue stained. M represents the molecular weight marker (kDa) used as standards and C represents control (fibrinogen only) incubated for 24 hour at 37 °C.

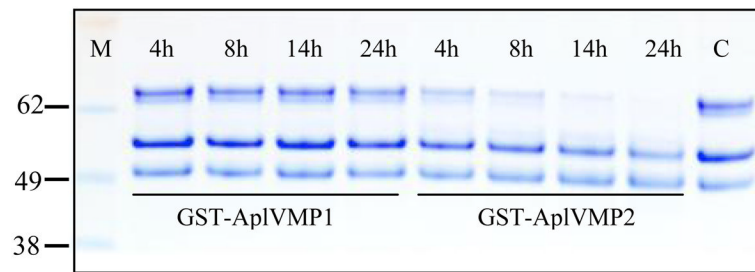


Fig. 6. Inhibition of AplVMP1 and AplVMP2-mediated proteolysis of fibrinogen. The same experiment conditions as described in Fig. 5 but addition of a final concentration of 10 mM EDTA and incubation from 4 to 24 h at 37 °C. Samples were collected at indicated time points and run on SDS gel under reducing condition and coomassie blue stained. M shows the standard protein marker (kDa). C indicates fibrinogen alone incubated at 37 °C for 24 hours.

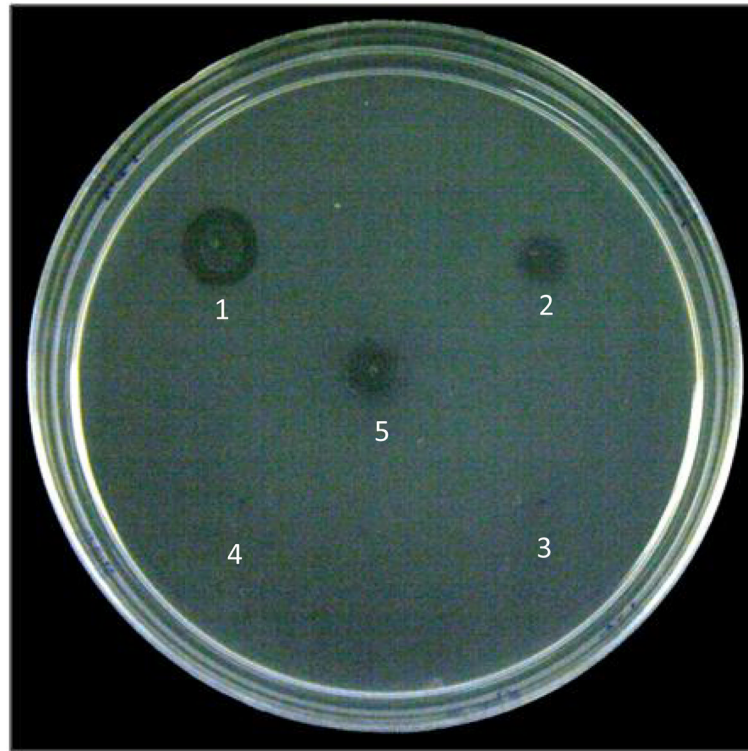


Fig. 7. Fibrinolytic activity of protein samples on a fibrin-agar plate. Samples were pipeted on plate and incubated at 37 °C for 24 hours. 1, GST-AplVMP2 (6 μ g); 2, GST-AplVMP1 (6 μ g); 3, GST (6 μ g); 4, GST elution buffer (20 μ l) and 5, crude venom(1.75 μ g). The fibrinolytic activities of AplVMP1, AplVMP2 and crude venom are 117U/mg, 167 U/mg and 457 U/mg, respectively on the basis of calculation described in materials and methods. GST alone and GST elution buffer are avoid of activity