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Molecular cloning and characterization of cDNAs encoding metalloproteinases from snake venom glands

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Abstract

Snake venom metalloproteinases (SVMPs) are a superfamily of zinc-dependent proteases and participate in a number of important biological, physiological and pathophysiological processes. In this work, we simultaneously amplified 9 cDNAs encoding different classes of metalloproteinases from glands of four different snake species (*Agkistrodon contortrix laticinctus*, *Crotalus atrox*, *Crotalus viridis viridis* and *Agkistrodon piscivorus leucostoma*) by RT-PCR with a pair of primers. Among the encoded metalloproteinases, two enzymes (*Ac/VMP-I* and *Ap/VMP-I*), three enzymes (*Ca/VMP-II*, *Cvv/VMP-II* and *Ap/VMP-II*) and four enzymes (*Ac/VMP-III*, *Ca/VMP-III*, *Cvv/VMP-III* and *Ap/VMP-III*) with the characteristic motif (HEXXHXXGXXH) of metalloproteinase belong to type P-I, P-II and P-III enzymes, respectively. Disintegrin domains of *Ca/VMP-II* and *Cvv/VMP-II* from two *Crotalus* snakes contain RGD-motif whereas *Ap/VMP-II* from *Agkistrodon* snake has KGD-motif. Instead of R/KGD-motif within disintegrin domain of SVMP-II enzyme *Ca/VMP-III*, *Cvv/VMP-III* and *Ap/VMP-III* enzymes contain SECD-motif, while *Ac/VMP-III* has DDGD-motif in their corresponding position of disintegrin-like domains. There are 12 Cys amino acids in cysteine-rich domains of each P-III enzyme. Moreover, a disintegrin precursor (*Ap/Dis*) with RGD motif also simultaneously amplified from the glands of *A.p.leucostoma* while amplifying *Ap/VMP-II* and *Ap/VMP-III*, which indicated that different types of SVMPs and related genes are present in a single species of snake and share a consensus sequence at the 3' and 5' untranslated regions. RT-PCR result also showed that P-III is highly expressed in *Crotalus* snakes than in *Agkistrodon* snakes. Aligning the deduced amino acid sequence of these enzymes with other SVMPs from GenBank database indicated that this is the first report on the isolation of cDNAs encoding P-II and P-III enzymes from *C.v.viridis* and *A.p.leucostoma* snakes. The availability of these SVMP sequences directly facilitated further studies of structure characterization and diversified function analysis.

Keywords

Snake venom metalloproteinase; RT-PCR; *Agkistrodon contortrix laticinctus*; *Crotalus atrox*; *Crotalus viridis viridis*; *Agkistrodon piscivorus leucostoma*

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1. Introduction

Hemorrhagic toxins are widely distributed in venoms of *Crotalidae* and *Viperidae* snakes. One of the key hemorrhagic toxins in snake venoms is snake venom metalloproteinases (SVMPs), the members of the repolysin subfamily of the M12 family of zinc-dependant proteases (Fox et al., 2005). SVMPs act synergistically with many other toxins to degrade the capillary basement membrane components like collagen type IV, laminin and fibronectin, and causes intense hemorrhage by acting locally and systemically (Baramova, et al., 1990; Bjarnason and Fox, 1994). SVMPs can also digest some blood coagulation factors including fibrinogen (Johnson and Ownby, 1993) and von Willebrand factor (Kamiguti et al., 1995), which increase the hemorrhagic effect. In addition to hemorrhage, SVMPs affect various biological, physiological and pathophysiological processes including thrombolysis, cancer metastasis, edema, hypotension, hypovolemia, inflammation, necrosis, coagulation cascade and fibrinolysis (Markland, 1998; Petretski et al., 2000; Matsui, et al., 2000)

SVMPs are synthesized in the venom gland as large multidomain proteins, which are generally classified into four main groups based on their amino acid domain structures (Hite et al., 1994; Bjarnason and Fox, 1994; Fox and Serrano, 2005). P-I: the smallest enzymes comprise a pro-domain and a metalloproteinase domain. A wide variety of P-I enzymes with various functions have been isolated from different snake species (Bello et al., 2006; Marcussi et al., 2007; Bernardes et al., 2008, Jia et al., 2009; Oliveira et al., 2009). P-II: the medium-size enzymes contain a pro-domain, a metalloproteinase and a disintegrin domain. To date, a number of P-II enzymes have been isolated from different snake species (Jeon and Kim, 1999; Fernandez et al., 2005; Singhamatr and Rojnuckarin, 2007, Jia et al., 2009). P-III: the most hemorrhagic toxins comprise a pro-domain, a metalloproteinase domain, a disintegrin-like domain and a cysteine-rich C-terminal domain. Although most information is available to SVMP-I and P-II, the number of cloned P-III enzymes is being increased and numerous P-III enzymes have been identified from different snake species (Assakura et al., 2003; Mazzi et al., 2004; Gay et al., 2005; Wan et al, 2006; Sanchez, et al., 2007; Azofeifa-Cordero, et al., 2008). P-IV: enzymes contain non-processed P-III structure and two C-type lectin-like polypeptides linked by a disulfide bridge to the metalloproteinase-containing polypeptide chain.

Although an increasing number of snake venom metalloproteinases (SVMPs) have now been identified as a result of cloning strategies, the diversity of SVMPs, coupled with their distinct function, prompts us to identify more SVMPs and characterize their specific structure and diversified functions. In the present study, we report the molecular cloning of 9 complete open reading frames (ORFs) of cDNAs encoding different metalloproteinases from four different species of snakes by RT-PCR with a pair of primers. These 9 cDNAs include two encoding P-I enzymes from snakes (*A.c.laticinctus* and *A.p.leucostoma*), three encoding P-II enzymes from snakes (*C.v.viridis*, *C.atrox* and *A.p.leucostom*) and four encoding P-III enzyme from snakes (*A. c. laticinctus*, *C. v. viridis*, *C. atrox*, *A. p. leucostom*,). In addition, a disintegrin precursor was also simultaneously amplified from glands of snake (*A. c. laticinctus*). The deduced amino acid sequences of each enzyme were characterized and the expression levels of cDNAs in corresponding snake was also presented.

2. Materials and methods

2.1 Animals

Four adult snakes (*Agkistrodon contortrix laticinctus*, *Agkistrodon piscivorus leucostoma*, *Crotalus atrox*, *Crotalus viridis viridis*) used in this study were maintained in the Natural Toxins Research Center Serpentarium, Texas A & M University-Kingsville, USA.

2.2 Extraction of total venom gland RNA

Four snakes (*A. c. laticinctus*; *C. v. viridis*; *A. p. leucostom*; *C. atrox*) were euthanized 3 days after venom extraction when DNA transcription rates reach maximum. Venom glands were collected from snakes and immediately frozen in liquid nitrogen. Two grams of venom glands was homogenized with pestle in mortar containing liquid N₂. Total RNA was extracted using TRIzol Reagent (Invitrogen, USA), and treated with RQ1 DNase (Promega) following the manufacturer's instructions to remove genomic DNA traces. RNA concentration was measured using Nanodrop spectrophotometer (Nanodrop Technologies, USA) after total RNA was frozen overnight in a - 80°C freezer, thaw and mixed completely.

2.3 RT-PCR

Five micrograms of total RNA extracted from venom gland was subjected to reverse transcription in a final volume of 20 µl using oligo-dT primer and SuperScript First-Strand Synthesis System for RT-PCR under conditions instructed by the manufacturer (Invitrogen, USA). One pair of primers, SVMP-IIIF, 5'-ccagccaaatccagcctccaaa-3' and SVMP-IIIR, 5'-tgcccatggagctttgtg-3', were designed on the basis of the consensus sequences of 3' and 5' untranslated regions (UTRs) of cDNAs encoding SVMP-III in GenBank database. The PCR reaction was carried out in a 50µl of reaction containing 25 ng of cDNA as a template, 1 × High Fidelity PCR buffer, 0.2 mM each dNTP, 2mM MgSO₄, 0.4 pmol each primer, 1.0 unit Platinum *Tag* High Fidelity (Invitrogen, USA), and autoclaved, distilled water up to 50 µl. PCR was performed using a thermal cycler (Gene Cyclor, BIO-RAD Hercules, CA) programmed for an initial denaturation (94°C for 1 min), followed by 30 cycles for 94°C for 30 s, 69°C for 30 s and 72°C for 3 min, and extension for 10 min at 72°C. The expression of 18S rRNA gene was used to assess the expression level of each cDNAs encoding SVMPs. PCR products were run on 1.2% agarose gel, and bands were excited and purified by GeneCleanIII according to the instruction manual. The purified PCR product was ligated to pGEM-T Easy Vector (Promega, USA), transformed into XL-blue competent cells (Stratage, USA) by heat-shock method.

2.4 Sequence analysis

Several white colonies from each ligation were picked to isolate plasmid DNA using GenElute plasmid Miniprep Kit (Sigma-Aldrich, USA). PCR with T7 forward and SP6 reverse primer was used to confirm the existence of inserts in plasmids. Three recombinant plasmid DNAs from each ligation were subjected to DNA sequence determination by using the BigDye terminator cycle sequencing mix (Applied Biosystems, USA) on an automated DNA sequencer (ABI Prism 310, Genetic Analyzer, Applied Biosystem). One sequence from two or three identical clones was used as the representative for further investigation. Multiple alignment of amino acid sequence was performed with Clustal W program (Thompson et al., 1994) and BOX shade. Protein domains were predicted with INTERPRO (<http://www.ebi.ac.uk/Tools/InterProScan/>). The phylogenetic tree constructed from the alignment was generated by a neighbor-joining (Saitou and Nei, 1987) algorithm in Lasergene 8.0 software (DNASTAR, Inc., Madison, WI, USA).

The sequences reported in this work were deposited in GenBank with the following accession numbers: *Ac/VMP-I* (GQ451436), *CaVMP-II* (GQ451438), *CvvVMP-II* (GQ451440), *Ap/VMP-II* (GQ451442), *Ac/VMP-III* (GQ451435), *CaVMP-III* (GQ451437), *CvvVMP-III* (GQ451439), *Ap/VMP-III* (GQ451441), *Ap/Dis*(GQ451443).

3. Results and discussion

3.1. RT-PCR amplification of SVMPs

SVMPs are the most potent hemorrhagic toxins with remarkable variations in snake venom and participate in a number of important biological, physiological and pathophysiological processes. In an attempt to improve our understanding of SVMPs, we chose four different snakes including two *Crotalus* snakes (*C. atrox*, *C.v. viridis*) and two *Agkistrodon* snakes (*A.c. laticinctus*, *A.p. leucostom*) for isolation of SVMPs. The total RNAs were extracted from venom glands and cDNAs were generated. The first-stranded cDNAs were used as the template in polymerase chain reactions (PCRs) with a pair of primers. Ten cDNA bands were amplified by two different PCR annealing temperatures (60 and 69 °C). Of these cDNA bands, four at approximately 2100 bp cDNAs from each snake gland, three 1800 bp cDNAs from three snakes (*C.atrox*, *C.v.viridis* and *A.p.leucostom*), one 1600-bp cDNA from *A.c.laticinctus* and one 600 bp cDNA from *A.p.leucostom* were amplified by an annealing temperature at 69 °C (upper panel in Fig.1). The cDNA of 1600 bp from *A.p.leucostom* was amplified by an annealing temperature of 60 °C (data not shown). It is noteworthy that 1600-bp and 1800-bp cDNAs were not able to be amplified with any annealing temperature (from 45–69 °C) from glands of *C.atrox* and *C.v.viridis*, and *A.c.laticinctus*, respectively.

After electrophoresis, RT-PCR products were excised from agarose gel and purified. The purified cDNAs were separately ligated into pGEM-T Easy vector (Promega, USA) according to the instructional manual. To avoid mutants made by PCR, we picked three independent cDNA clones from each ligation, and plasmid DNAs were isolated using Miniprep (Sigma, USA) and sequenced by normal procedures. Their nucleotide sequences were checked by both sequence and trace files. The amino acid sequences of the corresponding cDNAs were deduced by using Lasergene 8.0 (DNASTAR, Inc. Wisconsin, USA). A single representative cDNA sequence from two or three identical clones was used for subsequent investigation. Thus, all cDNA sequences reported herein were independently identified more than once, which verify that nucleotide changes did not result from errors during *Taq* DNA polymerase amplification.

Blastx searches using nucleotide sequences of 2100-bp, 1800-bp and 1600-bp as well as 600-bp cDNA against GenBank database revealed that these sequences have high sequence similarities with other SVMP enzymes except 600-bp cDNA which encodes disintegrin protein. The cDNAs of 1600 bp from *A.c. laticinctus* and *A.p. leucostoma* encode P-I enzyme. Thus, their predicted products were named *Ac/VMP-I* and *Ap/VMP-I*, respectively (Fig. 2). Three 1800-bp cDNAs from *C.atrox*, *C.v.viridis* and *A.p. leucostom* encode P-II enzyme and their predicted amino acids were called *CaVMP-II*, *CvvVMP-II* and *Ap/VMP-II*, respectively (Fig. 2). Four 2100-bp cDNAs from *A.c. laticinctus*, *C.atrox*, *C.v.viridis* and *A.p. leucostom* encode P-III enzyme and their predicted products were termed *Ac/VMP-III*, *CaVMP-III*, *CvvVMP-III* and *Ap/VMP-III*, respectively (Fig. 2). 600bp cDNA amplified from *A.p.leucostoma* encodes disintegrin proteins, thus its predicted amino acids was called *Ap/Dis* (Fig. 2).

To our knowledge, this is the first report of different members of SVMPs simultaneously amplified by a PCR reaction in single species of snake with a pair of primers, which indicated that the nucleotide sequences of 5' and 3' UTRs of different members of SVMPs as well as associated genes are conserved. The highly conserved 5' and 3' UTRs were also reported on SVMPs (Hite et al., 1994; Paine et al, 1994; Selistre de Araujo et al, 1995) as well as on phospholipase A₂ (Nakashima et al., 1995; Jia et al. 2008). There are no reports on identification of P-II enzymes from *A.c.laticinctus* to date, and we also were not able to amplify P-II from *A.c.laticinctus*. This implied that either P-II is not present in glands of *A.c.laticinctus* or if it is present, the nucleotide sequence of 5' and 3' UTRs of P-II differed

markedly from that of other SVMPs. Similarly, SVMP-I was not able to be amplified from glands of two *Crotalus* snakes. Three different enzymes (P-I, P-II and P-III) were amplified in gland of *A.p.leucostoma*, which indicated that different classes of SVMPs are co-expressed in single species of snake but they might synergistically affect different targets and adapt to the ecology of their prey. Identification of different classes of SVMPs from single species of snake was also demonstrated by others. Howes et al. (2003) identified P-I EoVMP1, P-III EoVMP2 and EoVMP3 from snake (*Echis ocellatus*). Oliveira et al. (2009) isolated HF3 (P-III) and BJ-PI (P-I) from *Bothrops jararaca* venom and at least three P-III class (HR1a, HR1b and HV1) and two P-I class (HR2a, H2-preteinase flavoridin precursor metalloproteinase) metalloproteinases were identified from the venom of *Trimeresurus flavoviridis* (Masuda et al., 2001; Kishimoto and Takahashi, 2002a, b).

In parallel with the constitutively expressed 18S rRNA (Lower panel in Fig. 1), RT-PCR results also show that the expression levels of each cDNA in glands of different snake species. Using equal amount of cDNAs from glands of different snakes as the template, RT-PCR result indicated that P-III and P-II enzymes are rich in *Crotalus* snakes, whereas the *Agkistrodon* snake have low P-III and P-II levels, which leads us to speculate that *Crotalus* snakes might be more hemorrhagic than *Agkistrodon* snakes. These results are also consistent with the previous studies showing that *Crotalus* snakes have lower minimal hemorrhagic doses (MHD) than those of *Agkistrodon* snakes (Sánchez et al., 2003a,2003b).

3.2 The SVMPs exhibit extensive sequence similarity or identity

The cDNA sequences of each enzyme were subjected to BlastX search for similarity or identity to other sequences in GenBank database, and results indicated that each cDNA sequence had a number of previously published SVMPs hits with homologies (63–99% identities). *AcIVMP-I* is 98 and 86% identical with P-I (2115195A) from *Agkistrodon contortrix laticinctus* (Selistre de Araujo et al., 1995) and *ApIVMP1* (FJ429179) from *Agkistrodon piscivorus leucostoma* (Jia et al., 2009), respectively. *ApIVMP-I* is identical to the *ApIVMP1* (FJ429179) which was isolated from cDNA library of venomous glands of *A.p.leucostoma* (Jia et al., 2009). Therefore, the sequence of *ApIVMP-I* was not submitted to GenBank. In addition, *ApIVMP-I* is 86% identical with P-I (2115195A) from *A.c.laticinctus* (Selistre de Araujo et al., 1995). *AcIVMP-I* and *ApIVMP-I* share a strong amino acid sequence similarity (86.1% identity).

CaVMP-II showed 81 and 84% homology with P-II (P0C6B6) from *Cryptelytrops albolabris* (Singhamatr et al., 2007) and P-II (AY071905) from *Gloydus halys*, respectively. *CvvVMP-II* is 88% identical with P-II (DQ464254) from *Sistrurus catenatus edwardsi* (Pahari et al., 2007) and 83% identical with P-II (AY204244) from *Gloydus saxatilis*. In an attempt to search the homology for *CvvVMP-II* from same species of snakes, there is no report on PII isolated from *C.v.viridis*. Therefore, as far as we know, this is the first report of cloning cDNA encoding P-II enzyme from *C.v.viridis*. *ApIVMP-II* shares high similarity (99 and 93% identity) with P-II (AB078906) from *Agkistrodon piscivorus piscivorus* (Okuda et al., 2002) and *ApIVMP2* (FJ429180) from *A.p. leucostoma* (Jia et al., 2009). *ApIVMP-II* and *ApIVMP2* were isolated from the same snake (*A.p.leucostoma*) glands using different methods. *ApIVMP2* was identified from cDNA library whereas *ApIVMP-II* was isolated by RT-PCR. In comparison with amino acid sequence of *ApIVMP-II*, *ApIVMP2* deletes 26 amino acids at the N-terminus and the rest of the two sequences are identical.

The Blastx search indicated that the predicted amino acid sequence of the 2100-bp cDNAs share a high degree of sequence similarity with other members of the SVMP-III family. *AcIVMP-III* showed 99, 83 and 79% homologies with P-III (U86634) from *A.c.laticinctus* (Selistre de Araujo et al., 1997), P-III (DQ464249) from *Sistrurus catenatus edwardsi* (Pahari et al., 2007) and P-III (AF450503) from *Bothrops erythromelas* (Silva, et al., 2003),

respectively. *CaVMP-III* is 98% identical with P-III (U21003) from *C.atrox* (zhou et al., 1995), and 97% identical with P-III (DQ164403) from *Crotalus durissus durissus* (Azofeifa-Cordero et al., 2008), respectively. *CvvVMP-III* showed a significant degree of homologies (99 and 97% identities) with PIIIs from *C. atrox* (U21003) and from *Crotalus durissus durissus* (DQ164403). There is no registered P-III identified from *C.v.viridis* in GenBank database to date. Therefore, *CvvVMP-III* might be a novel enzyme. As far as we know, *ApIVMP-III* is the first P-III cloned from *A.p. leucostoma* and its ORF is 71% identical with P-III (AY835996) from *Macrovipera lebetina* (Trummal et al., 2005) and 63% with P-III (DQ464251) from *Sistrurus catenatus edwardsi* (Pahari et al., 2007). The amino acid sequence of *CaVMP-III* and *CvvVMP-III* from two *Crotalus* snakes shares a high degree homology (99% identity), whereas that of *AcIVMP-III* and *ApIVMP-III* from *Agkistrodon* were 69.2% identical, which implied that the evolutionary relationship between *C.atrox* and *C.v.viridis* is closer than that between *A.p.leucostoma* and *A.c. laticinctus*. In addition, *AcIVMP-III* was found to have an additional nine amino acid sequence at the carboxyl terminus in comparison with other P-IIIs (Fig. 2).

The cDNA of 600 bp amplified from glands of *A.p.leucostoma* encodes 111 amino acids of disintegrin protein with theoretical molecular mass of 12,103 Da and isoelectric point (*pI*) value of 8.28, which is 99% identical with both disintegrin proteins isolated from *Agkistrodon piscivorus piscivorus* (AB078905), and *A.p.leucostroma* (EV854797) (Jia et al., 2008).

3.3 Amino acid structure analysis

A multi-domain pattern of these enzymes was recognized by INTERPRO program, which include an 18 residues of signal peptides, a pro-domain and a metalloproteinase domain for all enzymes. However, P-II enzymes including *CaVMP-II*, *CvvVMP-II* and *ApIVMP-II* encode one additional domain, a disintegrin domain on the carboxyl side of the metalloproteinase domain. P-III enzymes (*AcIVMP-III*, *CaVM-III*, *CvvVMP-III* and *ApIVMP-III*) encode two additional domains, a disintegrin-like domain and a carboxyl-terminal cysteine-rich domain (Fig. 2). The comparison of the amino acid sequences of these enzymes showed that they are very similar, which appears to be higher in the signal peptide and pro-domain sequences than in the mature proteins including metalloproteinase, disintegrin/disintegrin-like, cys-rich domains (Fig. 2). Likewise other members of SVMPs, the cysteine switch motif (PKMCGVT), zinc-binding motif (HEXXHXXGXXH) and methionine turn (CIM), and disintegrin motif RGD/KGD or disintegrin-like motif XXCD, are highly conserved among all types of SVMPs (Fig.2).

Both cDNAs of *AcIVMP-I* and *ApIVMP-I* encode an open reading frame (ORF) of 411 amino acids with a theoretical molecular mass of 46110.99 and 46608.07 Da and their *pI* values of 5.244 and 5.596, respectively. Both *AcIVMP-I* and *ApIVMP-I* consist of 18 residues of signal sequences at the N-terminus followed by pro-domains of 171 residues with cysteine switch motif (PKMCGVT), and metalloproteinase domain of 222 residues with zinc-binding motif HEXGHXXGXXHD involved in the stability of active site and the proteolytic activity of the enzyme (Bjarnason et al., 1994; Mazzi et al., 2004).

The cDNAs of three P-II enzymes (*CaVMP-II*, *CvvVMP-II* and *ApIVMP-II*) encode 478–486 amino acids of OPR with a predicted molecular mass of 53,813 – 54,736 Da and *pI* value of 4.90–5.22. The multi-domain pattern of these enzymes comprised 18 residues of signal sequence, a pro-domain residue with cysteine switch motif PKMCGVT, a metalloproteinase domain with zinc-binding motif HEXGHXXGXXHD, and a disintegrin domain with the integrin binding typical R/KGD-motif which enables these proteins to recognize integrin cell surface receptors (Kamiguti et al., 1998; Markland, 1998; Calvete et

al., 2005) (Fig. 2). The disintegrin domains of two *Crotalus* MP-IIs contain RGD-motif while that of *Agkistrodon* MP-II contains KGD-motif (Fig. 2).

The deduced amino acid sequences of four P-III enzymes (*Ac/VMP-III*, *CaVMP-III*, *CvvVMP-III* and *Ap/VMP-III*) consist of 609–620 amino acids, with a theoretical molecular mass of 68,212 – 69,466 Da and a pI value of 4.95–6.51. Like any other SVMP-III, the amino acid sequences of these enzymes contained a pro-domain, a metalloproteinase domain, a disintegrin-like domain and a C-terminal Cys-rich domain with 12 cys residues of amino acids structure (Fig. 2). The multi-domain structures of these enzymes are in accordance with the common precursor model of snake venom metalloproteinase/disintegrin-like/cysteine-rich domain (MDC) (Kini et al., 1992). Therefore, these enzymes were classified into the high molecular mass metalloproteinase, and belong to the P-III class of snake venom metalloproteinases. The disintegrin-like domains of *CaVMP-III*, *CvvVMP-III* and *Ap/VMP-III* enzymes have a SECD motif while *Ac/VMP-III* contains a DDGD-motif, which is the binding motif of various cell surface integrins (Markland, 1998; Calvete et al., 2005). The disintegrin-like domains of these P-III enzymes were similar to those of previously identified P-III enzymes (Kishimoto et al., 2002a; Cidade et al., 2006; Sanchez et al., 2007; Bello et al., 2006; Chen et al., 2008; Azofeifa-Cordero et al., 2008).

Disintegrins are low molecular weight non-enzymatic venom component containing RGD or KGD motifs reported as the main structures, which binds to platelet surface integrins and modulate platelet aggregation by different mechanisms (Kamiguti et al., 1996). The comparison of amino acid sequence of the disintegrin protein (*Ap/Dis*) isolated from *A.p.leucostoma* with sequences of SVMPs showed that they are more similar in the signal sequences and part of pro-domain than in the disintegrin domain (Fig. 2).

3.4. Phylogenetic study of SVMPs

Molecular phylogeny has been a powerful tool in classifying venom protein families and identifying distinct functional subtypes. Therefore, a phylogenetic tree was constructed to determine the evolutionary relations between these 9 SVMPs identified in this work and 13 other SVMPs selected from GenBank database, and categorized them into six distinct groups. Group 1 containing 6 P-II enzymes from different genera showed sequence similarity between 81.9 and 99.3%. Group 2 comprised 3 *Agkistrodon* P-I enzymes including *Ac/VMP-I*, *Ac/VMPI* and *Ap/VMP-I* shared sequence similarity between 86.1 and 98.3%. Group 3 represented by *CaVMP-II*, *CaVMP-II* and *GhVMP-II*. *CaVMP-II* and *CaVMP-II* isolated from the same species of snake but located in different places shared only 83% identity, which indicated the influence of geographical diversity. Group 4 contained *Ap/VMP-III* and *MiVMP-III* and shared comparatively low sequence similarity (72.3% identity) since these two P-III enzymes isolated from different genera. Group 5 was represented by four P-III enzymes (*Ac/VMP-III*, *Ac/VMP-III*, *SceVMP-III* and *BeVMP-III*) identified from three species of snakes, shared sequence similarity between 77.9 and 99.2%. Of these four P-III enzymes, *Ac/VMP-III* and *Ac/VMP-III*, isolated from two same species of snakes located in different geographic regions, contained 620 amino acids and shared an extraordinary degree of sequence conservation (99.2% identity). Group 6 contained 4 P-III enzymes including *CaVMP-III*, *CvvVMP-III*, *CaVMP-III* and *CddVMP-III* with 609 amino acids for each. All these enzymes were isolated from *Crotalus* snakes and showed a strong amino acid sequence similarity (between 97 and 99.2%). Their carboxyl terminal sequence (219 aa) showed a remarkable degree of sequence conservation (data not shown).

In conclusion, we cloned and characterized 9 cDNAs encoding SVMPs including four P-III, three P-II and two P-I enzymes from glands of four species of snakes by RT-PCR with a pair of primers. These enzymes have typical motifs in their corresponding domains in comparison with previously published SVMPs. P-III and P-II are highly expressed in venom

glands of *C.v.viridis* and *C.atrox*, but less expressed in *A. c. laticinctus* and *A.p. leucostom*. P-I enzymes were only isolated from *Agkistrodon* snakes. In addition, we simultaneously amplified a disintegrin cDNA from *A.p.leucostom* while amplifying SVMP cDNAs. The cDNA sequences of these enzymes showed considerable identity with each other and to analogues of related snakes. Our findings further confirmed that different types of SVMPs are present in single species of snake. These SVMP clones provide important information for further studies of the distinct structure-function of each enzymes, characterization of enzyme specificity by site-directed mutagenesis techniques as well as for studies of the action mechanism of venoms. Beyond these biological applications, these clones might have clinical applications, such as, coagulation research and diagnosis as well as design of metalloproteinase inhibitors and development of novel therapeutic agents.

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Abbreviations

SVMPs	snake venom metalloproteinases
RT-PCR	Reverse transcription polymerase chain reaction
cDNA	complementary DNA
UTRs	untranslated regions

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Fig.1.

Expression pattern of different metalloproteinase cDNAs in glands of different species of snakes. Ethidium bromide stained agarose gel showed that the relative quantitative RT-PCR was used to determine the expression of individual metalloproteinase cDNA relative to the constitutively expressed 18S rRNA. M, molecular marker; *Acl*, *Agkistrodon contortrix laticinctus*; *Ca*, *Crotalus atrox*; *Cvv*, *Crotalus viridis viridis*; *Apl*, *Agkistrodon piscivorus leucostoma*; P-III, P-II and P-I represent Snake venom metalloproteinase type I, type II and type III, respectively.

	Signal peptides	
CaVMP-III	MIQVLLVTICLAFFPYQGSSIIILESGNVNDYEVYPRKVTALPKGAVOPKYEDAMQYELKVNGEFVVLHLEKKNGLFSSD	80
CvvVMP-III	MIQVLLVTICLAFFPYQGSSIIILESGNVNDYEVYPRKVTALPKGAVOPKYEDAMQYELKVNGEFVVLHLEKKNGLFSSD	80
AcIVMP-III	MIQVLLVTICLAFFPYQGSSIIILESGNVNDYEVYPRKVTALPKGAVOPKYEDAMQYELKVNGEFVVLHLEKKNGLFSSD	80
ApIVMP-III	MIQVLLVTICLAFFPYQGSSIIILESGNVNDYEVYPRKVTALPKGAVOPKYEDAMQYELKVNGEFVVLHLEKKNGLFSSD	80
CaVMP-II	MIQVLLVTICLAFFPYQGSSIIILESGNVNDYEVYPRKVTALPKGAVOPKYEDAMQYELKVNGEFVVLHLEKKNGLFSSD	80
CvvVMP-II	MIQVLLVTICLAFFPYQGSSIIILESGNVNDYEVYPRKVTALPKGAVOPKYEDAMQYELKVNGEFVVLHLEKKNGLFSSD	80
AcIVMP-I	MIQVLLVTICLAFFPYQGSSIIILESGNVNDYEVYPRKVTALPKGAVOPKYEDAMQYELKVNGEFVVLHLEKKNGLFSSD	80
ApIVMP-I	MIQVLLVTICLAFFPYQGSSIIILESGNVNDYEVYPRKVTALPKGAVOPKYEDAMQYELKVNGEFVVLHLEKKNGLFSSD	80
ApIDis	MIQVLLVTICLAFFPYQGSSIIILESGNVNDYEVYPRKVTALPKGAVOPK-----	48
	Pro-domain	
CaVMP-III	YSETHYSPDGREITTYPLVEDHCYYHGRIENDADSTASISACNGLKGHFKLQEGMYLIEPLKLSDEAHAVYKYENVEKE	160
CvvVMP-III	YSETHYSPDGREITTYPLVEDHCYYHGRIENDADSTASISACNGLKGHFKLQEGMYLIEPLKLSDEAHAVYKYENVEKE	160
AcIVMP-III	YSETHYSPDGREITTYPLVEDHCYYHGRIENDADSTASISACNGLKGHFKLQEGMYLIEPLKLSDEAHAVYKYENVEKE	160
ApIVMP-III	YSETHYSPDGREITTYPLVEDHCYYHGRIENDADSTASISACNGLKGHFKLQEGMYLIEPLKLSDEAHAVYKYENVEKE	160
CaVMP-II	YSETHYSPDGREITTYPLVEDHCYYHGRIENDADSTASISACNGLKGHFKLQEGMYLIEPLKLSDEAHAVYKYENVEKE	160
CvvVMP-II	YSETHYSPDGREITTYPLVEDHCYYHGRIENDADSTASISACNGLKGHFKLQEGMYLIEPLKLSDEAHAVYKYENVEKE	160
AcIVMP-I	YSETHYSPDGREITTYPLVEDHCYYHGRIENDADSTASISACNGLKGHFKLQEGMYLIEPLKLSDEAHAVYKYENVEKE	160
ApIVMP-I	YSETHYSPDGREITTYPLVEDHCYYHGRIENDADSTASISACNGLKGHFKLQEGMYLIEPLKLSDEAHAVYKYENVEKE	160
ApIDis	YSETHYSPDGREITTYPLVEDHCYYHGRIENDADSTASISACNGLKGHFKLQEGMYLIEPLKLSDEAHAVYKYENVEKE	48
CaVMP-III	DEAPKMGCVTON-WESYEPIKKASQIVVTAHQ-KYNPFREVELVIVVDKAMVTKNNGNLDIKIRMYEIVNTVNEIYR	238
CvvVMP-III	DEAPKMGCVTON-WESYEPIKKASQIVVTAHQ-KYNPFREVELVIVVDKAMVTKNNGNLDIKIRMYEIVNTVNEIYR	238
AcIVMP-III	DEAPKMGCVTON-WESYEPIKKASQIVVTAHQ-KYNPFREVELVIVVDKAMVTKNNGNLDIKIRMYEIVNTVNEIYR	239
ApIVMP-III	DEAPKMGCVTON-WESYEPIKKASQIVVTAHQ-KYNPFREVELVIVVDKAMVTKNNGNLDIKIRMYEIVNTVNEIYR	240
CaVMP-II	DEAPKMGCVTON-WESYEPIKKASQIVVTAHQ-KYNPFREVELVIVVDKAMVTKNNGNLDIKIRMYEIVNTVNEIYR	238
CvvVMP-II	DEAPKMGCVTON-WESYEPIKKASQIVVTAHQ-KYNPFREVELVIVVDKAMVTKNNGNLDIKIRMYEIVNTVNEIYR	237
AcIVMP-I	DEAPKMGCVTON-WESYEPIKKASQIVVTAHQ-KYNPFREVELVIVVDKAMVTKNNGNLDIKIRMYEIVNTVNEIYR	237
ApIVMP-I	DEAPKMGCVTON-WESYEPIKKASQIVVTAHQ-KYNPFREVELVIVVDKAMVTKNNGNLDIKIRMYEIVNTVNEIYR	237
ApIDis	DEAPKMGCVTON-WESYEPIKKASQIVVTAHQ-KYNPFREVELVIVVDKAMVTKNNGNLDIKIRMYEIVNTVNEIYR	48
	Metalloproteinase	
CaVMP-III	LYIHVALVGLIWSNEDKIVKPEAGYTTNAFGWRRKIDLLRRKHSHDNAQLLTAIDLR-VGLAYVGSVCHPKRSTGII	317
CvvVMP-III	LYIHVALVGLIWSNEDKIVKPEAGYTTNAFGWRRKIDLLRRKHSHDNAQLLTAIDLR-VGLAYVGSVCHPKRSTGII	317
AcIVMP-III	LYIHVALVGLIWSNEDKIVKPEAGYTTNAFGWRRKIDLLRRKHSHDNAQLLTAIDLR-VGLAYVGSVCHPKRSTGII	319
ApIVMP-III	LYIHVALVGLIWSNEDKIVKPEAGYTTNAFGWRRKIDLLRRKHSHDNAQLLTAIDLR-VGLAYVGSVCHPKRSTGII	320
CaVMP-II	LYIHVALVGLIWSNEDKIVKPEAGYTTNAFGWRRKIDLLRRKHSHDNAQLLTAIDLR-VGLAYVGSVCHPKRSTGII	318
CvvVMP-II	LYIHVALVGLIWSNEDKIVKPEAGYTTNAFGWRRKIDLLRRKHSHDNAQLLTAIDLR-VGLAYVGSVCHPKRSTGII	317
AcIVMP-I	LYIHVALVGLIWSNEDKIVKPEAGYTTNAFGWRRKIDLLRRKHSHDNAQLLTAIDLR-VGLAYVGSVCHPKRSTGII	317
ApIVMP-I	LYIHVALVGLIWSNEDKIVKPEAGYTTNAFGWRRKIDLLRRKHSHDNAQLLTAIDLR-VGLAYVGSVCHPKRSTGII	317
ApIDis	LYIHVALVGLIWSNEDKIVKPEAGYTTNAFGWRRKIDLLRRKHSHDNAQLLTAIDLR-VGLAYVGSVCHPKRSTGII	48
CaVMP-III	QDYSEINLVAVVMAHEHGHNLGINHDGSGYCSGCDYACIMRPEISPEPSTFFSNCSYFCWDFINNNPBCILNEPLGTD	397
CvvVMP-III	QDYSEINLVAVVMAHEHGHNLGINHDGSGYCSGCDYACIMRPEISPEPSTFFSNCSYFCWDFINNNPBCILNEPLGTD	397
AcIVMP-III	QDYSEINLVAVVMAHEHGHNLGINHDGSGYCSGCDYACIMRPEISPEPSTFFSNCSYFCWDFINNNPBCILNEPLGTD	399
ApIVMP-III	QDYSEINLVAVVMAHEHGHNLGINHDGSGYCSGCDYACIMRPEISPEPSTFFSNCSYFCWDFINNNPBCILNEPLGTD	400
CaVMP-II	QDYSEINLVAVVMAHEHGHNLGINHDGSGYCSGCDYACIMRPEISPEPSTFFSNCSYFCWDFINNNPBCILNEPLGTD	398
CvvVMP-II	QDYSEINLVAVVMAHEHGHNLGINHDGSGYCSGCDYACIMRPEISPEPSTFFSNCSYFCWDFINNNPBCILNEPLGTD	397
AcIVMP-I	QDYSEINLVAVVMAHEHGHNLGINHDGSGYCSGCDYACIMRPEISPEPSTFFSNCSYFCWDFINNNPBCILNEPLGTD	397
ApIVMP-I	QDYSEINLVAVVMAHEHGHNLGINHDGSGYCSGCDYACIMRPEISPEPSTFFSNCSYFCWDFINNNPBCILNEPLGTD	397
ApIDis	QDYSEINLVAVVMAHEHGHNLGINHDGSGYCSGCDYACIMRPEISPEPSTFFSNCSYFCWDFINNNPBCILNEPLGTD	49
	Disintegrin-like domain	
CaVMP-III	IISEPPVCG-NELLEVGECDCGTFENCONECDDAATCKLISGSCQCHGDCCHQCKFSKSGTECRASMS-EDPAHCTGQS	476
CvvVMP-III	IISEPPVCG-NELLEVGECDCGTFENCONECDDAATCKLISGSCQCHGDCCHQCKFSKSGTECRASMS-EDPAHCTGQS	476
AcIVMP-III	IISEPPVCG-NELLEVGECDCGTFENCONECDDAATCKLISGSCQCHGDCCHQCKFSKSGTECRASMS-EDPAHCTGQS	478
ApIVMP-III	IISEPPVCG-NELLEVGECDCGTFENCONECDDAATCKLISGSCQCHGDCCHQCKFSKSGTECRASMS-EDPAHCTGQS	479
CaVMP-II	IISEPPVCG-NELLEVGECDCGTFENCONECDDAATCKLISGSCQCHGDCCHQCKFSKSGTECRASMS-EDPAHCTGQS	473
CvvVMP-II	IISEPPVCG-NELLEVGECDCGTFENCONECDDAATCKLISGSCQCHGDCCHQCKFSKSGTECRASMS-EDPAHCTGQS	476
AcIVMP-I	IISEPPVCG-NELLEVGECDCGTFENCONECDDAATCKLISGSCQCHGDCCHQCKFSKSGTECRASMS-EDPAHCTGQS	476
ApIVMP-I	IISEPPVCG-NELLEVGECDCGTFENCONECDDAATCKLISGSCQCHGDCCHQCKFSKSGTECRASMS-EDPAHCTGQS	476

Fig. 2. Multiple alignment of amino acids sequence of SVMPs. The alignment was generated with the Clustal W program. The different domains of each enzyme were indicated by arrows. The cysteine-switch motifs are single lined and the consensus sequence of the zinc-binding sites are double lined while the disintegrin or disintegrin-like motifs are indicated with asterisks. Abbreviations: VMP-III, -II and I represent venom metalloproteinase III, II and I, respectively; *Dis*, disintegrin; *Ca*, *Crotalus atrox*; *Cvv*, *Crotalus viridis viridis*; *Acl*, *Agkistrodon contortrix laticinctus*; *Apl*, *Agkistrodon piscivorus leucostoma*.

Fig. 3.

Phylogenetic relationships of SVMPs based on their amino acid sequences alignment. This neighbor-joining analysis was used to categorize these sequences into six distinct groups. Branch lengths are drawn to scale and represent the percent amino acid divergence for each group or sequence. The genes in bold were cloned from this work and the others were selected from previously published papers. *ApVMP2* (FJ429180) from *Agkistrodon piscivorus leucostoma*. *AppVMP-II* (AB078906) from *Agkistrodon piscivorus piscivorus*. *SceVMP-II* (DQ464254) from *Sistrurus catenatus edwardsi*. *GsVMP-II* (AY204244) from *Gloydus saxatilis*. *AcVMP-I* (2115195A) from *Agkistrodon contortrix laticinctus*. *CaVMP-II* (P0C6B6) from *Cryptelytrops albolabris*. *GhVMP-II* (AY071905) from *Gloydus halys*. *MIVMP-III* (AY835996) from *Macrovipera lebetina*. *AcVMP-III* (U86634) from *Agkistrodon contortrix laticinctus*. *SceVMP-III* (DQ464249) from *Sistrurus catenatus edwardsi*. *BeVMP-III* (AF450503) from *Bothrops erythromelas*. *CaVMP-III* (U21003) from *Crotalus atrox*. *CddVMP-III* (DQ164403) from *Crotalus durissus durissus*.