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Review

Snake Venom Metalloproteinases (SVMPs): A structure-function update

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ABSTRACT

Snake venom metalloproteinases (SVMPs) represent a diverse group of multi-domain proteins with several biological activities such as the ability to induce hemorrhage, proteolytic degradation of fibrinogen and fibrin, induction of apoptosis and inhibition of platelet aggregation. Due to these activities, SVMPs are responsible for many of the well-known pathological phenotypes in snake envenomations caused particularly by species from the *Viperidae* family and the *Crotalinae* subfamily. These proteins have been classified based on their size and domain structure into P–I, P-II and P-III classes. Comparatively, members of the P–I SVMPs possess the simplest structures, formed by the catalytic metalloproteinase domain only; the P-II SVMPs are moderately more complex, having the canonical disintegrin domain in addition to the metalloproteinase domain; members of the P-III class are more structurally varied, comprising the metalloproteinase, disintegrin-like, and cysteine-rich domains. Proteolytic cleavage, repeated domain loss and presence of other ancillary domains are responsible for structural diversities in the P-III class. However, studies continue to unveil the relationship between the structure and function of these proteins. In this review, we recovered evidences from literature on the structural peculiarities and functional classification of Snake Venom Metalloproteinases. In addition, we reflect on diversities that exist among each class while taking into account specific and up-to-date class-based activities.

1. Snake Venom Metalloproteinases (SVMPs)

Snake Venom Metalloproteinases (SVMPs) are zinc-dependent enzymes that have been described in the venoms of snakes, particularly from species in the *Viperidae* family and *Crotalinae* subfamily (Fox and Serrano, 2009, 2008a, 2005). Additionally, the distribution of SVMPs can be extended to the venoms of *Elapidae*, *Atractaspididae* and *Colubridae* snake families, although in these families, SVMPs are found in smaller concentrations (Ching et al., 2006; Georgieva et al., 2011; Kulkeaw et al., 2007; Quinton et al., 2005; Tan et al., 2019, 2015). Recent studies have showed that the presence of SVMPs in certain venoms could be related to environmental features such as the climate, which in turn influences snake foraging strategies (Vidal et al., 2019; Zancolli et al., 2019). Snakes that live in an environment with milder winters and less seasonal variation in temperature have a haemotoxic venom mostly composed by SVMPs. These data indicate that epigenetic factors influence the evolution of toxins, having an impact in the field of snake genomics and consequently affecting the relationship between SVMPs structures and its functions.

Features such as variation in domain composition, functional diversity and possibility of class recombination of SVMPs have frustrated previous attempts to resolve and harmoniously incorporate newly discovered SVMPs into the known classes. For instance, the initial classification of SVMPs divided them into four classes based on the cDNA/mRNA sequences of their precursors (N-classification or Nucleotide-based classification) or the size of the mature proteins purified from the snake venoms (P-classification or Protein-based classification) (Bjarnason and Fox, 1994; Hite et al., 1994). The N–I or Metalloproteinase class I has the simplest sequence, which is encoded by a signal sequence, a pro-domain and a metalloproteinase domain. The N–I transcripts resulted in the P–I class of proteins that had only the metalloproteinase domain. The N-II class encoded a signal, a pro-domain, a metalloproteinase and a disintegrin domains giving rise to the mature proteins of the P-II class (metalloproteinase and disintegrin domains). Differently, the N-III class encoded a disintegrin-like domain instead of the disintegrin domain and an additional cysteine-rich domain. The N-III nucleotide sequences resulted in the mature proteins of the P-III class. The last class, the N-IV, encompassed

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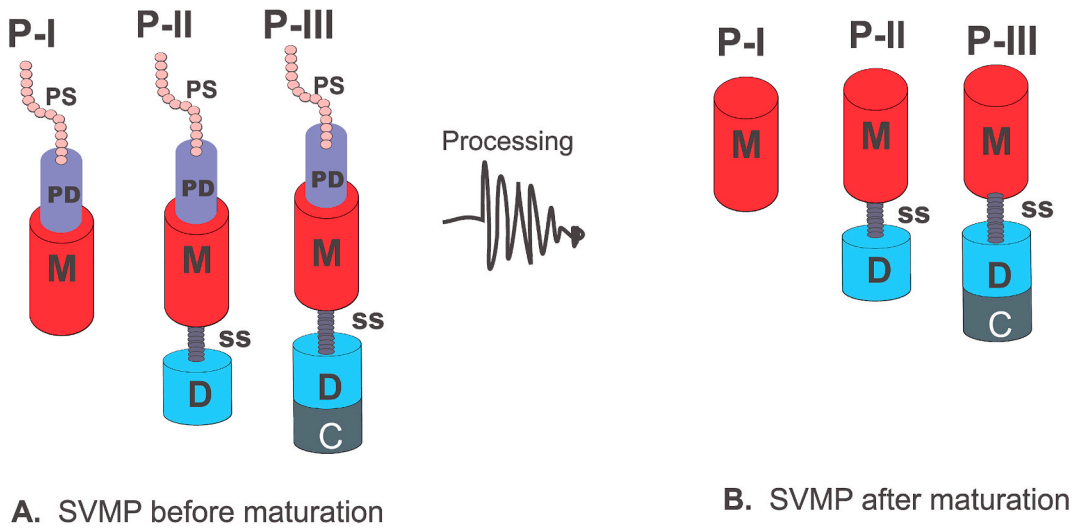

Fig. 1. A general scheme of the canonical structures peculiar to each SVMP class. **1A** depicts immature SVMPs and it encompasses a P–I class containing peptide signal sequence (PS), pro-domain (PD) and metalloproteinase (M) domain; P-II consist of PS, PD, M, spacer sequence (SS) and disintegrin (D) domain; and P-III contains PS, PD, M, SS, disintegrin-like (D) and cysteine rich (C) domains. **1B** delineates processed SVMPs (without PS and PD). Thus, canonical structure of the matured P–I consists of only the M domain; P-II contains M, SS and D domains; and P-III is comprised of M, SS, D and C domains.

an additional lectin-like domain, giving rise to P-IV proteins (Bjarnason and Fox, 1994; Hite et al., 1994). However, N-IV transcripts have never been found in the transcriptomes of snake venom glands, so this classification is no longer used (Bjarnason and Fox, 1994; Moura-da-Silva et al., 2011).

The most adopted SVMP classification dates from 2008 and it classifies the metalloproteinases into three classes: P–I (20–30 kDa) containing a metalloproteinase domain in its mature form; P-II (30–60 kDa; P-IIa, P-IIb, P-IIc, P-IId and P-IIe) containing a disintegrin domain linked to the C-terminus of the metalloproteinase domain, and P-III (60–100 kDa; P-IIIa, P-IIIb, P-IIIc and P-IIId) consisting of a metalloproteinase domain, a disintegrin-like and a cysteine rich domains (Fox and Serrano, 2008b). The lack of proteomic evidence and the non-observation of a P-IV mRNA transcript led to the merge of the P-IV class into the P-III class (P-IIId) with the suggestion that the P-IV class represents a post translational modification of the accepted P-III structure with the addition of a lectin-like domain (Fox and Serrano, 2008b, 2009; Markland and Swenson, 2013). Fig. 1 shows a general scheme of the canonical structures peculiar to each SVMP class before and after maturation.

1.1. SVMPs classification

1.1.1. The P–I SVMPs

The P–I class includes the smallest and simplest metalloproteinases with molecular masses ranging from 20 to 30 kDa and the presence of only a metalloproteinase (M) domain when isolated from the snake venom (Markland and Swenson, 2013; Sani et al., 2019). The M domain has a conserved zinc-binding sequence (HEXXHXXGXXH) followed by a conserved "Methionine-turn" motif (Da Silva et al., 2012; Herrera et al., 2015). P–I members have the ability to degrade basement membrane components, which corresponds to their proteolytic, hemorrhagic and edema forming activities (Preciado et al., 2019; Suvilesh et al., 2017). However, as P–I SVMPs only have the M domain, studies demonstrated that they have a diffuse localization in the extracellular matrix, and consequently a less toxic effect than P-II or P-III SVMPs (Suvilesh et al., 2017).

Many studies have characterized proteins belonging to the P–I class, including their crystal structures (Akao et al., 2010; Bernardes et al., 2013; Chou et al., 2013; Ferreira et al., 2009; Gomis-Rüth et al., 1994, 1993; Gong et al., 1998; Huang et al., 2002; Kumasaka et al., 1996; Lingott et al., 2009; Lou et al., 2005; Patiño et al., 2010; Watanabe et al., 2009; Zhang et al., 1994). Shannon et al. (1989) and Takeya et al. (1989) were the first to report the complete sequences of two representatives of the P–I class: Ht-d and H_2-proteinase (Shannon et al., 1989; Takeya et al., 1989). Early characterization of Ht-d, a hemorrhagic metalloproteinase from the venom of the western diamond-black rattlesnake *Crotalus atrox*, showed that this P–I representative has a molecular mass of 23,234 Da (Shannon et al., 1989). In the same year, structural analysis of peptides resulting from digests with cyanogen bromide, lysyl endopeptidase, trypsin, *Staphylococcus aureus* V8 protease and thermolysin revealed the primary structure and the position of the disulfide linkage in the non-hemorrhagic H_2-proteinase from habu snake (*Trimeresurus flavoviridis*) (Takeya et al., 1989). The same study also demonstrated that the P–I H_2-proteinase is a non-glycosylated single chain polypeptide with an approximate size of 23 kDa (22,991 Da) and 201 amino acid residues.

In general, P–I members present high similarity in their structures, but a wide variation regarding their hemorrhagic activity (Camacho et al., 2019). Data from molecular dynamic simulations showed differences in the structures of hemorrhagic and non-hemorrhagic P–I SVMPs (Wallnoefer et al., 2010). The main difference was associated with the flexibility and rigidity of the loop near the active site. Briefly, non-hemorrhagic P–I members have great flexibility in the loop region after the Met-turn whereas hemorrhagic P–I members have higher flexibility in the loop area before the Met-turn. This differential flexibility can be responsible for the unequal hemorrhagic activity found in this class, having a role in protein-protein interaction at the basement membrane (Camacho et al., 2019; Wallnoefer et al., 2010).

An extensive review that punctuated the structure and biological activity of SVMPs mentioned the characterization of the crystal structures of 10 members of the P–I class: Atrolysin C, Acutolysin A, BapI, Fibrolase, HT-2, Atroxase, LHF-II, H_2-proteinase, HR2A and Grammihelysin I; whose biological activities include hemorrhage, mionecrosis, inflammation, fibrinolysis, non-hemorrhagic proteolysis, and apoptosis (Fox and Serrano, 2005). After this review, other P–I members were characterized such as the non-hemorrhagic FII from *Agkistrodon acutus* (Lou et al., 2005), the non-hemorrhagic Leucurolysin-A from *Bothrops leucurus* (Ferreira et al., 2009), Batx-I from *Bothrops atrox* (Patiño et al., 2010), BpirMP from *Bothrops pirajai* (Bernardes et al., 2013), the hemorrhagic okinalysin from *Ovophis okinavensis* (Komori et al., 2014), the fibrinolytic and hemorrhagic proteins jararafibrase III (21,400 ± 500 Da) and jararafibrase IV (21,200 ± 400 Da) from the venom of *Bothrops*

Table 1
P–I SVMPs representatives and their reported activities.

	SVMP (P–I)	Molecular Mass (kDa)	Snake Species	Reported Activities	References
1.	HR2A	23.02	*Trimeresurus flavoviridis*	Hemorrhagic	Miyata et al. (1989); Takahashi and Ohsaka (1970)
2.	HT-2	25	*Crotalus ruber*	Hemorrhagic and fibrinogenolytic	Mori et al. (1987)
3.	HT-3	25.50	*Crotalus ruber*	Hemorrhagic and fibrinogenolytic	Mori et al. (1987)
4.	Atroxase	23.50	*Crotalus atrox*	Non-hemorrhagic and fibrinolytic	Willis and Tu (1988)
5.	Ht-d	23.23	*Crotalus atrox*	Hemorrhagic (degradation of fibronectin, laminin, type IV collagen, nidogen, and gelatins)	Shannon et al. (1989); Uniprot
6.	H_2-proteinase	22.99	*Trimeresurus flavoviridis*	Non-hemorrrhagic and proteolytic	Takeya et al. (1989)
7.	LHF-II	22.3	*Lachesis muta*	Hemorrhagic, caseinolytic fibrinolytic and fibrinogenolytic	Sanchez et al. (2016)
8.	Jararafibrase III	21.4	*Bothrops jararaca*	Fibrinolytic and hemorrhagic	Maruyama et al. (1993)
9.	Jararafibrase IV	21.2	*Bothrops jararaca*	Fibrinolytic and hemorrhagic	Maruyama et al. (1993)
10.	Adamalysin II	24	*Crotalus adamenteus*	Endopeptidase, non-hemorrhagic, and inactivates serpins by limited proteolysis of their reactive-site loops	Gomis-Rüth et al. (1993); Uniprot
11.	BapI	24	*Bothrops asper*	Weakly hemorrhagic, fibrinogenolytic, caseinolytic and fibrinolytic	Gutiérrez et al. (1995)
12.	Acutolysin A	22	*Agkistrodon acutus*	Hemorrhagic	Gong et al. (1998)
13.	GramminelysinI	27.02	*Trimeresurus gramineus*	Fibrinogenolytic and apoptotic	Wu et al. (2001)
14.	EoVMP1	24	*Echis ocellatus*	Non-hemorrhagic and activates prothrombin (procoagulant)	Howes et al. (2003, 2005)
15.	Agkislysin	22	*Agkistrodon acutus*	Fibrinogenolytic, fibrinolytic and prothrombin-activating	Wang et al. (2004)
16.	Insularinase	22.64	*Bothrops insularis*	Fibrinogenolytic, fibrinolytic and prothrombin-activating	de Albuquerque Modesto et al. (2005)
17.	FII	26 kDa	*Agkistrodon acutus*	Non-hemorrhagic	Lou et al. (2005)
18.	BjussuMP-II	24	*Bothrops jararacussu*	Antiplatelet, non-hemorrhagic, gelatinolytic, collagenolytic, fibrinolytic and fibrinogenolytic	Marcussi et al. (2007)
19.	BnP1	24	*Bothrops neuwendi*	Fibrinolytic and fibrinogenolytic	Baldo et al. (2008)
20.	Bothrojararactivase	22.83	*Bothrops jararaca*	Prothrombin-activating	Berger et al. (2008)
21.	Bj-PI	~ 25	*Bothrops jararaca*	Fibrinogenolytic and caseinolytic	Oliveira et al. (2009)
22.	Leucurolysin-A	23	*Bothrops leucurus*	Non-hemorrhagic and non-glycosylated-fibrinogenase	Ferreira et al. (2009)
23.	CcH1	25	*Cerastes cerastes*	Hemorrhagic	Boukhalfa-Abib et al. (2009)
24.	Batx-1	23.3	*Bothrops atrox*	Fibrinogenolytic and Hemorrhagic	Patiño et al. (2010)
25.	Bj-PI2	28.08	*Bothrops jararaca*	Non-hemorrhagic and fibrinolytic. It can recruit inflammatory cells	Da Silva et al. (2012)
26.	BpMP-I	20	*Bothropoides pauloensis*	Non-hemorrrhagic and fibrinogenolytic	Naves de Souza et al. (2012)
27.	BpirMP	23.1	*Bothrops pirajai*	Thrombocytic, fibrinogenolytic and fibrinolytic	Bernardes et al. (2013)
28.	BmooMPα-I	22.6	*Bothrops moojeni*	Kininogenase	Okamoto et al. (2014)
29.	Okinalysin	22	*Ovophis okinavensis*	Caseinolytic and hemorrhagic	Komori et al. (2014)
30.	BpMP-II	23	*Bothropoides pauloensis*	Azocaseinolytic. It can inhibit cell adhesion and angiogenesis	Aché et al. (2015)
31.	Bar-I	23.39	*Bothrops barnetti*	Non-hemorrhagic, fibrinogenolytic and fibrinolytic	Sanchez et al. (2016)

jararaca (Maruyama et al.), bothrojararactivase (22, 829 Da), BJ-PI (~ 25 kDa) and Bj-PI2 (28 kDa) also from *B. jararaca* venom (Berger et al., 2008; Da Silva et al., 2012; Oliveira et al., 2009). Bothrojaractivase stands out due to its pronounced prothrombin-activating activity, while BJ-PI displays high fibrinogenolytic and caseinolytic effect. Bj-PI2 lacks myonecrotic and hemorrhagic activity although it has the ability to recruit inflammatory cells and degrade fibrin (Berger et al., 2008; Da Silva et al., 2012; Oliveira et al., 2009). In another study, two P–I SVMPs, BpMP-I and BpMP-II, were characterized from the venom of *Bothrops pauloensis*. The non-hemorrhagic BpMP-I is a 20 kDa P–I that exhibits fibrinogenolytic activity while BpMP-II (23 kDa) was observed to inhibit cell adhesion and angiogenesis (Naves de Souza et al., 2012). Although there was no positive correlation between the presence of BpMP-II and local tissue hemorrhage, its azocaseinolytic activity has also been reported (Aché et al., 2015). Table 1 describes a list of the most well-known P–I SVMPs.

1.2. The P-II SVMPs

The P-II class is structurally more complex than the P–I class but less than those of the P-III class. Several studies from the cDNA sequences have shown that the P-II class contains multiple domains including a pro-domain (PD; non-matured proteins), a metalloproteinase (M) domain and a disintegrin (D) domain (Camacho et al., 2014; Chen et al., 2003; Fox and Serrano, 2009, 2008b; 2005; Nikai et al., 2000; Takeda et al., 2012). The derived P-II members have been only purified from the venoms of snakes belonging to the *Viperidae* family, which suggests that they emerged from a P-III SVMP precursor that lost its cysteine-rich domain, after the divergence of *Viperidae* from the *Elapidae* family (Carbajo et al., 2015; Sanz and Calvete, 2016). Additionally, the disintegrin domain of P-II SVMPs may have been lost on diverse moments, forming the P–I SVMPs (Casewell et al., 2011).

P-II class members have molecular masses ranging from 30 to 60 kDa and a number of proteins belonging to this class have already been described (Casewell et al., 2011). Although the arrangement of the domains is the same in all the P-II members, there is a variation in the length of the spacer sequence between the metalloproteinase and disintegrin domains and in the pattern of cysteines. P-II SVMPs possess 12 cysteine residues that are uniquely patterned, but some members such as agkistin (Wang et al., 2003) and albolamin (Jangprasert and Rojnuckarin, 2014) have two additional cysteine residues. A report in the literature revealed that the disintegrin domain, which abutted the C-terminal segment of the metalloproteinase domain could be vulnerable to proteolytic release (Hite et al., 1992). However, this proteolytic processing could be prevented by the presence of 2-additional cysteine residues capable of forming disulfide bonds, which gives stability to the

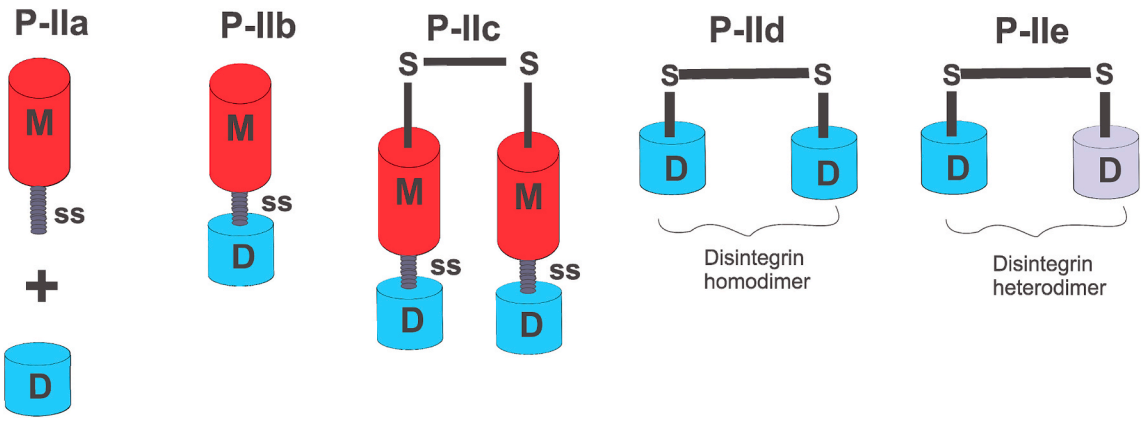

Fig. 2. Sub-classification of matured P-II SVMPs. The P-IIa consists of a processed fragment of metalloproteinase (M) and another fragment of disintegrin (D). The P-IIb is canonical and it is comprised of M, spacer sequence (SS) and D domain. Members of the P-IIc sub-class are dimers; each monomer is a canonical form. Dimerization of two identical disintegrins give rises to the P-IId sub-class; whereas in the P-IIe, non-identical disintegrins form dimers.

structure, thereby hindering auto-proteolysis of the C-terminal segment of the metalloproteinase domain (Fox and Serrano, 2005). Additionally, the pattern of cysteine in different domains of P-II SVMPs can be responsible for their diversities as such pattern, in addition to stability, may contribute to unique folding and dimerization. As previously noted, the current classification scheme (Fig. 2) recognizes 5 sub-classes of P-II SVMPs including P-IIa, P-IIb, P-IIc, P-IId and P-IIe (Fox and Serrano, 2008b).

One unique feature of the members of the P-II class is the presence of an integrin-binding motif usually called RGD-motif (RGD stands for arginine-glycine-aspartate). The RGD-motif is not totally conserved in all P-II members. There are copious reports of RGD variants such as KGD (lysine-glycine-aspartate), VGD (valine-glycine-aspartate), WGD (tryptophan-glycine-aspartate), MLD (methionine-leucine-aspartate), RTS (arginine-threonine-serine), KTS (lysine-threonine-serine) and so on (Calvete et al., 2005; Cesar et al., 2019; Rivas-Mercado and Garza-Ocañas, 2017). The RGD-motif is embedded in a flexible loop constituted by the oligopeptide chain of 13 aminoacyl residues, which interact significantly with the integrin $\alpha_{IIB}\beta_3$/GpIIb/IIIa inhibiting platelet aggregation (Chung et al., 1999; Masuda et al., 2000).

In some P-II structures, additional intra-chain interaction culminate to structural reinforcement and stability, thus, preventing the proteolytic release of their disintegrins. Sequence alignment and phylogenetic analysis revealed this feature in jerdonitin, a 36 kDa protein purified from the venom of *Trimeresurus jerdonii* (Chen et al., 2003). Additionally, the presence of the RGD-motif in jerdonitin enhanced its ability to inhibit ADP-induced human platelet aggregation in a dose-dependent fashion (Chen et al., 2003). In another report, two P-II proteins, TJM-1 and TJM-2, were isolated from the venom of *Trimeresurus jerdonii* and comparative analysis has revealed the presence of additional Cys-407 and Cys-426 in TJM-2. More recently, Jangprasert and Rojnuckarin (2014) reported the cloning of albolamin from a cDNA library obtained from the green pit viper *Cryptelytrops albolaris* venom gland (Jangprasert and Rojnuckarin, 2014). Western blot probe using anti-polyhistidine antibody revealed that the recombinant protein has a molecular mass of approximately 35 kDa and it belongs to the P-II class SVMP. In addition to the conserved RGD-integrin binding motif present in the disintegrin domain, albolamin also contains two additional cysteine residues in its structure, which may prevent auto-proteolysis of the protein (Jangprasert and Rojnuckarin, 2014). Activity report showed that albolamin could degrade human collagen type IV and inhibit collagen-induced platelet aggregation in a dose dependent manner (Jangprasert and Rojnuckarin, 2014).

Continuous advancements in recombinant DNA technology have provided scientists with tools for the characterization of more protein structures. For instance, there was the successful cloning of the ahpfibrase from the venom of *Gloydius halys* (Zhang et al., 2010). This P-II protein has 32 kDa and contains the canonical RGD-motif, which is responsible for platelet aggregation inhibition, besides its fibrinolytic activity (Zhang et al., 2010). In addition, Wang et al. (2003) cloned the P-II agkistin from the venom of *Agkistrodon halys* and demonstrated that this protein of 57 kDa (as shown by Western blot profiling) inhibited ADP-induced platelet aggregation and induced apoptosis in Human Uterine Microvascular Endothelial Cells (HUMEC) (Wang et al., 2003). Sequence determination and structural analysis revealed the presence of the RGD motif in the disintegrin domain of agkistin and the presence of two cysteine residues (Cys 407 and Cys 426) in addition to the 12 uniquely patterned cysteine residues of P-II members (Wang et al., 2003).

As mentioned previously, there are several reports showing that some members of the P-II SVMP class do not contain the canonical RGD motif (Calvete et al., 2005; Cesar et al., 2019; Rivas-Mercado and Garza-Ocañas, 2017). While some motifs share similarities, others are too varied and since the RGD motif is responsible for inhibition of platelet aggregation, members lacking this motif may have different activities and functions. For example, the primary structure of bilitoxin-1, a P-II SVMP of 32 kDa from *Agkistrodon bilineatus*, revealed that this hemorrhagic protein lacks platelet aggregation inhibitory activity and this may be due to the presence of a MGD binding sequence contrary to the canonical RGD motif (Nikai et al., 2000). Notwithstanding, the MGD motif has been shown to impair the function of $\alpha_5\beta_1$ integrin (Calvete et al., 2005). On the other hand, peptide mass fingerprinting and protein sequence revealed that the presence of the KGD motif in the disintegrin domain of stejnitin did not affect its ability to inhibit ADP-induced platelet aggregation (Han et al., 2007). This 35 kDa protein was purified from the venom of *Trimeresurus stejnegeri*. DNA fragmentation alongside flow cytometry analysis demonstrated that it could trigger apoptosis in ECV304 cells (Han et al., 2007). Sighamatr and Rojnuckarin (2007) reported the isolation of the recombinant P-II albolatin from the venom of *Trimeresurus albolaris*. Expression analysis of the D domain showed the presence of a KGD motif that does not interfere with the inhibition of collagen-induced platelet aggregation (Singhamatr and Rojnuckarin, 2007). In a similar case, the dimeric BlatH1 (84 kDa), isolated from *Bothriechis lateralis*, hydrolyzed fibrinogen, gelatin and azocasein and it inhibited both ADP- and collagen-induced platelet aggregation (Camacho et al., 2014). In fact, this protein lacks the conventional RGD-integrin-binding motif, which was replaced by the TDN (threonine-aspartate-asparagine) motif (Camacho et al., 2014). CcMP-II, a 35 kDa hemorrhagic P-II protein purified from *Cerastes cerastes* snake venom, has the ability to inhibit platelet aggregation and it has been shown to hydrolyze ECM components such as type IV collagen and laminin, besides possessing α-fibrinogenase activity (Boukhalfa-Abib

Table 2
P-II SVMP members and their reported activities.

	SVMP (P-II)	M Mass (kDa)	Domain Composition (As Studied)	Snake Species	Integrin-binding Motif	Reported Activities	References
1.	Trigamin	9	Disintegrin	*Trimeresurus gramineus*	RGD	Inhibition of platelet aggregation	Huang et al. (2002); Neeper and Jacobson (1990)
2.	Salmosin 1	7.5	Disintegrin	*A. h. brevicandus*)	RGD	Inhibition of $\alpha_{IIb}\beta_3$ integrin binding to fibrinogen	Kang et al. (1998); Park et al. (1998)
3.	Atrolysin E/D	7.4	Disintegrin	*Crotalus atrox*	MVD	Inhibition of both ADP- and collagen-induced platelet aggregations	Shimokawa et al. (1997)
4.	Bilitoxin-1	32.28	Dimeric; each monomer contains metalloproteinsase, and disintegrin	*Agkistrodon bilineatus*	MGD	Hemorrhagic	Nikai et al. (2000)
5.	Jerdonitin	36	Metalloproteinase and disintegrin	*Trimeresurus jerdonii*	RGD	Inhibition of ADP-induced human platelet aggregation	Chen et al. (2003)
6.	Agkistin	~57	Metalloproteinase and disintegrin.	*Agkistrodon halys*	RGD	Inhibition of ADP-induced platelet aggregation and induction of apoptosis in HUMEC	Wang et al. (2003)
7.	Jerdonin	7.5	Disintegrin	*Trimeresurus jerdonii*	RGD	Antagonist of the platelet's GPIIb-IIIa receptor	Zhou et al. (2004)
8.	Bitistatin	9	Disintegrin	*Bothrops arietans*	RGD	Inhibition of platelet aggregation via binding to the $\alpha_{IIb}\beta_3$ integrin	Knight and Romano (2005)
9.	Bothrostatin	8	Disintegrin	*Bothrops jararaca*	RGD	Inhibition of collagen-induced platelet aggregation	Fernandez et al. (2005)
10.	Stejnitin	35	Metalloproteinase and disintegrin	*Trimeresurus stejnegeri*	KGD	Inhibition of ADP-induced platelet aggregation and induction of apoptosis in ECV304 cells	Han et al. (2007)
11.	Albolatin (only the disintegrin domain)	11	Homodimeric disintegrins	*Trimeresurus albolaris*	KGD	Inhibition of collagen-induced platelet aggregation	Singhamatr and Rojnuckarin (2007)
12.	Dis*Ba*-01	8	Disintegrin	*Bothrops alternatus*	RGD	$\alpha_v\beta_3$ – blocking effect and inhibition of angiogenesis in HUVEC	Danilucci et al. (2019); Ramos et al. (2008)
13.	Ahpfibrase	32	Metalloproteinase and disintegrin	*Gloydius halys*	RGD	Fibrinolytic activity and inhibition of platelet aggregation	Zhang et al. (2010)
14.	Insularin	14	Disintegrin	*Bothrops insularis*	RGD	Inhibition of endothelial cell adhesion	Della-Casa et al. (2011)
15.	CamVMPII (r-cam-dis)	8	Disintegrin	*Crotalus adamanteus*	RGD	Inhibition of both ADP- and collagen-induced platelet aggregation	Suntravat et al. (2013)
16.	Albolamin	35	Metalloproteinase and disintegrin	*Cryptelytrops albolaris*	RGD	Degradation of human collagen type IV and inhibition of collagen-induced platelet aggregation	Jangprasert and Rojnuckarin (2014)
17.	BlatH1	84	Dimeric; each monomer contains metalloproteinsase, and disintegrin	*Bothriechis lateralis*	RGD	Inhibition of both ADP- and collagen-induced platelet aggregation; hemorrhagic, fibrinogenolytic, gelatinolytic and azocaseinolytic	Camacho et al. (2014)
18.	BnMPIIx (neuwiedin)	8.18	Disintegrin	*Bothrops neuwiedi*	RGD	Inhibition of ADP-induced platelet aggregation	Lima-dos-Santos et al. (2015)
19**0.**	CcMP-II Boukhalfa-	35	Metalloproteinsase, and disintegrin	*Cerastes*		Hemorrhagic and α-fibrinogenase activity	Boukhalfa-Abib and Laraba-Djebari (2015)

and Laraba-Djebari, 2015).

Several studies have cloned and expressed the disintegrin domain of P-II SVMPs. For instance, Dis*Ba*-01 (Ramos et al., 2008), bothrostatin (Fernandez et al., 2005), BnMPIIx (neuwiedin) (Lima-dos-Santos et al., 2015) and CamVMPII (r-cam-dis) (Suntravat et al., 2013) were identified from cDNA libraries constructed from the venom transcriptome of four different snake species: *Bothrops alternatus, Bothrops jararaca, Bothrops neuwiedi and Crotalus adamanteus*, respectively. SDS-PAGE analyses showed that bothrostatin, CamVMPII and Dis*Ba*-01 are proteins of approximately 8 kDa (Ramos et al., 2008). Although MALDI-TOF analysis showed that BnMPIIx has a molecular mass of 8 kDa, data from SDS-PAGE fingerprint correlated this protein to a molecular mass of 14 kDa (Lima-dos-Santos et al., 2015). It was demonstrated that BnMPIIx inhibited ADP-induced platelet aggregation whereas bothrostatin inhibited collagen-induced platelet aggregation. CamVMPII inhibited both processes (Suntravat et al., 2013). The inability of BnMPIIx to inhibit collagen-induced platelet aggregation may be due to a unique cysteine sequence and the substitution of some residues at the C-terminal of its RGD binding loop. Dis*Ba*-01, a recombinant RGD-disintegrin, demonstrated a $\alpha_v\beta_3$ integrin blocking effect (Ramos et al., 2008). In fact, recent data from Danilucci et al. (2019) demonstrated that Dis*Ba*-01 interfered in the $\alpha_v\beta_3$/VEGFR2 crosstalk, culminating into inhibition of HUVEC adhesion, migration and VEGF-mediated angiogenesis (Danilucci et al., 2019). Other examples of cloned disintegrins containing a RGD-motif are: trigamin (*Trimeresurus gramineus*), a platelet aggregation inhibitor (Neeper and Jacobson, 1990), jerdonin (*Trimeresurus jerdonii*), an antagonist of the platelet GPIIb-IIIa receptor (Zhou et al., 2004), bitistatin (*Bothrops arietans*), a platelet aggregation inhibitor that binds to the $\alpha_{IIb}\beta_3$ integrin ((Knight and Romano, 2005), insularin (*Bothrops insularis*), an inhibitor of endothelial cell adhesion (Della-Casa et al., 2011) and salmosin 1 (*A. h. brevicandus*), that inhibited the binding of $\alpha_{IIb}\beta_3$ integrin to fibrinogen (Kang et al., 1998; Park et al., 1998). A summary of P-II SVMPs members is described in Table 2.

1.3. The P-III SVMPs

The P-III class of SVMP encompasses a group of diverse proteins with higher molecular masses, usually ranging from 60 to 100 kDa and having a more complex structure than P–I and P-II SVMPs (Markland

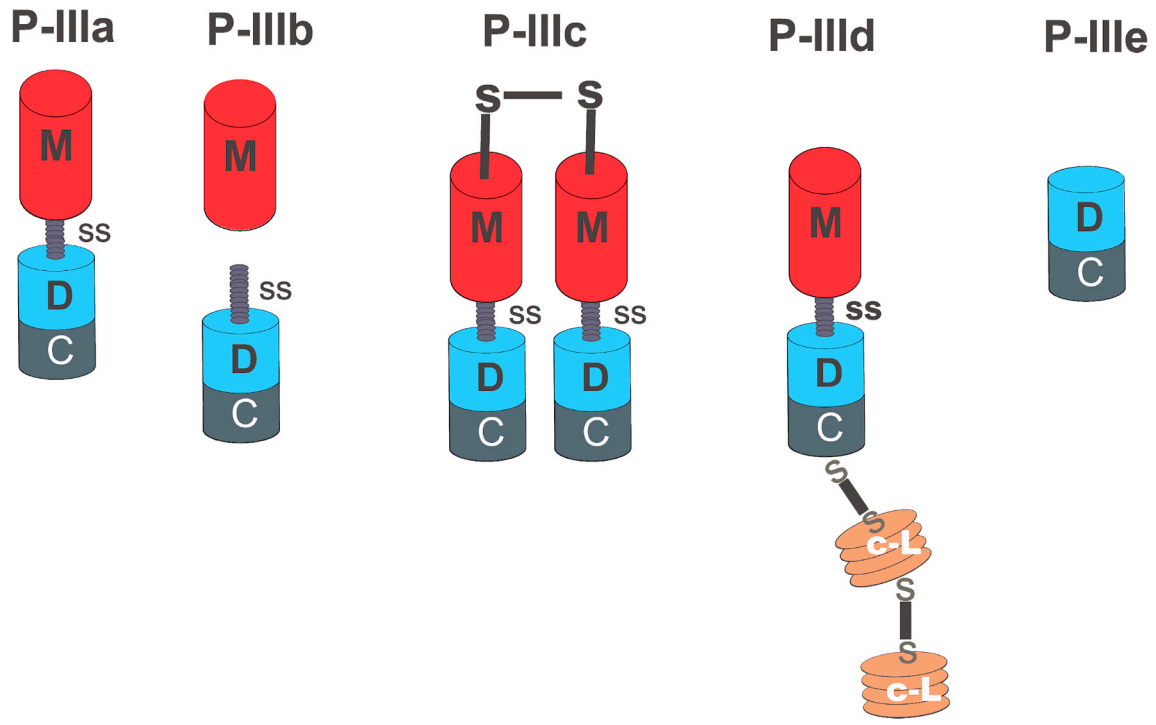

Fig. 3. Classification of the P-III SVMPs. P-IIIa has a metalloproteinase (M), a disintegrin-like (D) and a cysteine-rich (C) domain, displaying the canonical structure of P-III SVMPs. The D adjacent to the M domain of P-IIIb subclass is vulnerable to proteolytic cleavage. Members of the P-IIIc subclass form homo- or heterodimers connected by a disulfide bridge formed between the M domains of the two monomers. The P-IIId subclass represents a complexed P-III SVMP in which the C-terminal of the cysteine-rich domain is covalently linked to a tandem arrangement of two covalently bridged Snake C-type lectin-like regions-Snaclecs (c-L). The M domain is absent in the novel P-IIIe subclass and the processed form is a didomain containing D and C domains.

and Swenson, 2013). Members of the P-III class are widely distributed in the venoms of the *Viperidae*, *Elapidae*, *Atractaspididae* and *Colubridae* families (Casewell et al., 2011), supporting the studies that indicate that P-III SVMPs are derived from an early recruitment, duplication and neofunctionalization of an ancestral ADAM gene, before the radiation of the advanced snakes (~ 60 million years ago) (Carbajo et al., 2015; Casewell, 2012; Fry, 2005). P-III class comprise a pro-domain (PD), metalloproteinase (M), disintegrin-like (D) and cysteine-rich (C) domains. The presence of the non-catalytic disintegrin-like domain and the cysteine rich domain in the P-III class accounts for their potent hemorrhagic activities (Escalante et al., 2011). For instance, VaH4 is a hemorrhagin purified from the venom of *Vipera ammodytes ammodytes*. This protein is extremely hemorrhagic – hydrolyzing extracellular matrix components such as fibronectin, nidogen and the α-chain of fibrinogen – and possesses a potent cytotoxic activity against HeLa cells (Leonardi et al., 2014).

Due to its structural complexity, the P-III class was further classified into four subclasses: P-IIIa, P-IIIb, P-IIIc and P-IIId (Fox and Serrano, 2008b). The P-IIIa is considered the canonical subclass and it encompasses members containing a metalloproteinase, disintegrin-like and cysteine rich domains after post-translational exclusion of its pro-domain (Fox and Serrano, 2008b). A representative member of this subclass is the protein ohagin, a 50 kDa fibrinogenolytic protease isolated from the venom of *Ophiophagus Hannah* (Guo et al., 2007). The P-IIIb subclass comprises proteins that had undergone post-translational cleavage and/or proteolytic processing, which releases a mature form containing a disintegrin-like domain (with a D/ECD motif) and a cysteine-rich domain Fox and Serrano (2008b); Markland and Swenson (2013). A representative member of this subclass is the protein Alternagin isolated from the venom of *Bothrops alternatus* (Souza et al., 2000). Alternagin inhibits $\alpha_2\beta_1$ integrin, thereby decreasing cell adhesion to type I collagen (Selistre-de-Araujo et al., 2005) and interferes with the $\alpha_2\beta_1$/VEGFR2 crosstalk, thus inhibiting angiogenesis in HUVECs (dos Santos et al., 2020). Other example of the P-IIIb subclass is the protein TSV-DM purified from *Trimeresurus stejnegeri* venom wich potentially induces apoptosis on vascular endothelial cells (Wan et al., 2006a). In the P-IIIc subclass of SVMPs, some members are able to form dimers. VaH3 is a P-IIIc hemorrhagin purified from the venom of *Vipera ammodytes ammodytes* (Sajevic et al., 2013). Data from MALDI/TOF analysis suggest that it has a molecular mass of 104 kDa in its non-reduced form, while it has a molecular mass of 53.7 kDa following chemical reduction by S-carbamoylmethylation (Sajevic et al., 2013). VaH3 cleaves basement membrane (BM) and extracellular matrix (ECM) proteins including type IV collagen, fibronectin and nidogen (Sajevic et al., 2013). This data with the report of Leonardi et al. (2014) suggest that members of the P-IIIc class may be proteolytically potent against basal lamina and ECM proteins, although VaH3 can also cleave prothrombin and factor X without activating them (Leonardi et al., 2014; Sajevic et al., 2013). In addition, the dimers formed by the P-IIIc subclass can be constituted by two homologous monomers as in the case of VaH3 (Sajevic et al., 2013) or by a heterodimer (Wan et al., 2006b). Complexation of some P-III class members with C-type lectin-like protein (Snaclecs) has been identified in the P-IIId subclass. In this subclass, a C-type lectin like domain is connected to the canonical P-III structure by a disulfide bridge (Bjarnason and Fox, 1995). RVV-X is a representative of the P-IIId subclass and it can be found in the Russel's viper venom (*Daboia russelii*). RVV-X sequence was completely reported by Takeya et al. (1992) (Takeya et al., 1992). The unique 3D-structure of RVV-X has a hook-spanner-wrench configuration capable of binding to membrane receptors and coagulation factors instead of carbohydrates, demonstrating how important the relationship between structure and function is (Takeda et al., 2007). Moreover, RVV-X potently inhibited both ADP- and collagen-induced platelet aggregations, thereby causing intravascular coagulation in preys (Takeya et al., 1992). The complex 3D-structures of PIIIc and P-IIId members are related to enhanced pharmacological activity and toxicity (Doley and Kini, 2009).

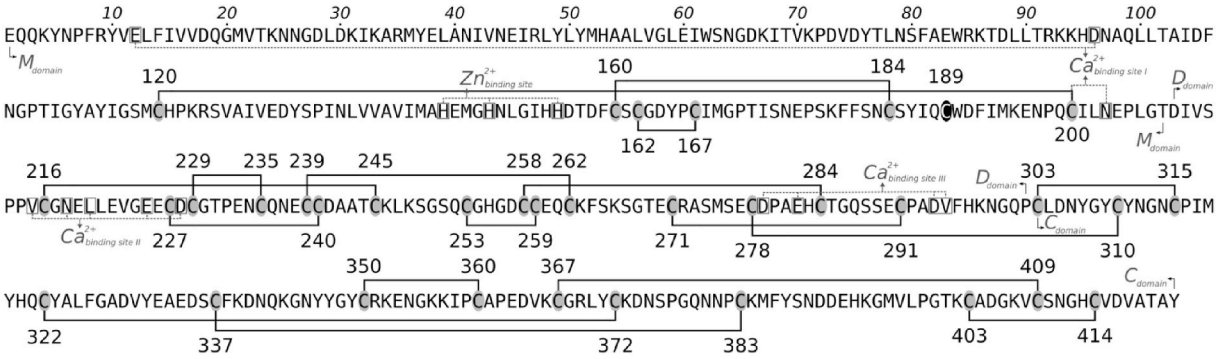

Fig. 4. Amino acid sequence of bothropasin, a PIII isolated from *B. jararaca* venom (Muniz et al., 2008). Cys residues that are in the form of disulfide bonds are shaded gray, numerated and connected by continuous lines. The free cysteine 189 is shaded in black. Calcium-binding sites as well as zinc-binding site are indicated and connected by dashed lines. Metalloproteinase (M), disintegrin (D) and cysteine-rich (C) domains.

The four subclasses (P-IIIa, b, c, and d) are well-known and documented in the literature. However, studies suggested the existence of a P-IIIe subclass comprising proteins that lack the catalytic metalloproteinase domain (Balija et al., 2020; Leonardi et al., 2019). Although Casewell et al. (2011) had previously correlated the evolutionary history of serpents to a repeated domain loss (Casewell et al., 2011), recent data from proteomic and transcriptomic analysis of *Vipera ammodytes ammodytes* venom identified Vaa-MPIII-3 as a member of the new P-IIIe

Table 3
Sub-classification of P-III SVMPs and their activities.

	SVMP	Sub-class	Domains in processed forms	Snake Species	Activities	References
1.	Ohagin	P-IIIa	Metalloproteinase, disintegrin-like and cysteine rich domains	*Ophiophagus hannah*	Fibrinogenolytic activity and inhibition of ADP-induced platelet aggregation	Guo et al. (2007)
2.	VaF1	P-IIIa	Metalloproteinase, disintegrin-like and cysteine rich domains	*Vipera ammodytes ammodytes*	α-fibrinogenase activity	Leonardi et al. (2015)
3.	HR-Ele1	P-IIIa	Metalloproteinase, disintegrin-like and cysteine rich domains	*Protobothrops elegans*	Hemorrhagic, αβ-fibrinogenase, type I collagenase	Oyama and Takahashi (2015)
4.	Atrolysin A	P-IIIa	Metalloproteinase, disintegrin-like and cysteine rich domains	*Crotalus atrox*	Hemorrhagic, proteolytic, degrades plasma fibronectin, type-I collagenase, type-IV collagenase, fibrinogenase, gelatinase, sheddase of glycoprotein VI,	Fox and Bjarnason (1995); Pinto et al. (2007)
5.	Albocollagenase	P-IIIa	Metalloproteinase, disintegrin-like and cysteine rich domains	*Cryptelytrops albolaris*	Inhibition of collagen-induced platelet aggregation, type IV collagenase	Pinyachat et al. (2011)
6.	Alternagin-C	P-IIIb	Disintegrin-like domain linked to a cysteine-rich domain	*Bothrops alternatus*	Inhibition of integrin-collagen interaction, inhibition of angiogenesis in HUVECs	Cominetti et al. (2004); dos Santos et al. (2020); Selistre-de-Araujo et al. (2005); Souza et al. (2000)
7.	Catrocollastatin	P-IIIb	Disintegrin-like domain linked to a cysteine-rich domain	*Crotalus atrox*	Inhibition of collagen-induced platelet aggregation	Fox and Serrano (2008); Zhou et al. (1995)
8.	Baltergin-C	P-IIIb	Disintegrin-like domain linked to a cysteine-rich domain	*Bothrops alternatus*	Hemorrhagic, type IV collagenase	Gay et al. (2009)
9.	SV-PAD-2	P-IIIc	Dimeric; each monomer contains: a metalloproteinase, a disintegrin-like and a cysteine-rich domain	*Protobothrops elegans*	αβγ-fibrinogenase inhibition of ADP- and collagen-induced inhibition of platelet aggregation	Oyama and Takahashi (2015)
10.	AHPM	P-IIIc	Dimeric; each monomer contains: a metalloproteinase, a disintegrin-like and a cysteine-rich domain	*Agkistrodon halys pallas*	α-fibrinogenase, inhibition of ADP-induced platelet aggregation	Song et al. (2013)
11.	VaH3	P-IIIc	Dimeric; each monomer contains: a metalloproteinase, a disintegrin-like and a cysteine-rich domain	*Vipera ammodytes ammodytes*	Proteolytic cleavage of prothrombin, X factor as well as BM and ECM protein components	Sajevic et al. (2013)
12.	VaH4	P-IIIc	Dimeric; each monomer contains: a metalloproteinase, a disintegrin-like and a cysteine-rich domain	*Vipera ammodytes ammodytes*	Hemorrhagic (hydrolysis of ECM proteins) and cytotoxic activities against Hela cells	Leonardi et al. (2014)
13.	HV1	P-IIIc	Dimeric; each monomer contains: a metalloproteinase, a disintegrin-like and a cysteine-rich domain	*Trimeresurus flavoviridis*	Apoptosis of vascular endothelial cell *in vitro*	Masuda et al. (2001)
14.	VAP1	P-IIIc	Dimeric; each monomer contains: a metalloproteinase, a disintegrin-like and a cysteine-rich domain	*Crotalus atrox*	Fibrinogenolytic, Apoptosis of vascular endothelial cell *in vitro*	Masuda et al. (2000)
15.	VLFXAs	P-IIId	Metalloproteinase, disintegrin-like, cysteine-rich and two C-type lectin-like domains	*Vipera ammodytes ammodytes*	Activation of coagulation factor X to Xa, inhibition of collagen-induced platelet aggregation	Leonardi et al. (2008)
16.	RVV-X	P-IIId	Metalloproteinase, disintegrin-like, cysteine-rich and two C-type lectin-like domains	*Daboia russelli*	Inhibition of both ADP- and collagen-induced platelet aggregations	Takeya et al. (1992)
17.	Vaa-MPIII-3	P-IIIe	Disintegrin-like and cysteine-rich domains	*Vipera ammodytes ammodytes*	Hemorrhagic	Leonardi et al. (2019)

Table 4
Three-dimensional structures of SVMPs available in the Protein Data Bank (PDB).

Protein (PDB)	Snake Species	Class SVMP	Method	References
Acutolysin A (1BUD)	*Deinagkistrodon acutus*	P-I	X-ray	Gong et al. (1998)
Acutolysin-C (1QUA)	*Deinagkistrodon acutus*	P-I	X-ray	Zhu et al. (1999)
FII (1YP1)	*Deinagkistrodon acutus*	P-I	X-ray	Lou et al. (2005)
AaHIV (3HDB)	*Deinagkistrodon acutus*	P-III	X-ray	Zhu et al. (2009)
Disintegrin (2M75)	*Calloselasma rhodostoma*	P-IIa (D-domain)	NMR	Chuang et al. (2013)
Rhodostomin (Rho) (2M7F, 2M7H, 4R5R, 3UCI, 4RQG, 4M4C, 4R5U, 14Y, 1Q7I, 1Q7J, 2LJV, 2M75, 2PJF, 2PJI)	*Calloselasma rhodostoma*	P-II (recombinant D-domain with and without mutations)	NMR and X-ray	Chen et al. (2009); Huang et al. (2015); Shiu et al. (2015)
Atragin (3K7L)	*Naja atra*	P-III	X-ray	Guan et al. (2010)
K-like protein (3K7N)	*Naja atra*	P-III	X-ray	Guan et al. (2010)
Leucurolysin-A (4Q1L)	*Bothrops leucurus*	P-I	X-ray	Ferreira et al. (2015)
TM-1 (4J4M)	*Protobothrops mucrosquamatus*	P-I	X-ray	Chou et al. (2013)
BmooMPalpha-I (3GBO)	*Bothrops moojeni*	P-I	X-ray	Akao et al. (2010)
TM-3 complexed with pEKW (1KUK)	*Protobothrops mucrosquamatus*	P-I	X-ray	Huang et al. (2002)
TM-3 (1KUF)	*Protobothrops mucrosquamatus*	P-I	X-ray	Huang et al. (2002)
Bothropasin (3DSL)	*Bothrops jararaca*	P-III	X-ray	Muniz et al. (2008)
VAP1 (2ERQ, 2ERP, 2ERO))	*Crotalus atrox*	P-III	X-ray	Takeda et al. (2006)
VAP2 (2DW0, 2DW1, 2DW2)	*Crotalus atrox*	P-III	X-ray	Igarashi et al. (2007)
Disintegrin (1TEJ)	*Echis carinatus*	P-IId	X-ray	Bilgrami et al. (2005)
Acostatin (3C05)	*Agkistrodon contortrix contortrix*	P-IIe	X-ray	Moiseeva et al. (2008)
Schistatin (1RMR)	*Echis carinatus*	P-IId	X-ray	Bilgrami et al. (2004)
Trimestatin (1JL2)	*Protobothrops flavoviridis*	P-IIa (D-domain)	X-ray	Fujii et al. (2003)
Bitistatin A (2MOP)	*Bitis arietans*	P-IIa (D-domain)	NMR	Carbajo et al. (2015)
Bitistatin B (2MP5)	*Bitis arietans*	P-IIa (D-domain)	NMR	Carbajo et al. (2015)
Salmosin (1L3X)	*Gloydius brevicaudus*	P-IIa (D-domain)	NMR	Shin et al. (2003)
Echistatin (1RO3)	*Echis carinatus*	P-IIa (D-domain)	NMR	Monleón et al. (2005)
Obtustatin (1MPZ)	*Macrovipera lebetina obtusa*	P-IIa (D-domain)	NMR	Moreno-Murciano et al. (2003)
Kistrin (1N4Y)	*Calloselasma rhodostoma*	P-IIa (D-domain)	NMR	Adler et al. (1991)
Jerdostatin (2W9V, 2W9O, 2W9U, 2W9W)	*Protobothrops jerdonii*	P-IIa (recombinant D-domain)	NMR	Carbajo et al. (2011)
RVV-X (2E3X)	*Daboia siamensis*	P-III	X-ray	Takeda et al. (2007)
Atrolysin-C (1HTD/1ATL)	*Crotalus atrox*	P-I	X-ray	Zhang et al. (1994)
BaP1 (1ND1)	*Bothrops asper*	P-I	X-ray	Watanabe et al. (2009)
BaP1 complexed with a peptidomimetic (2W14)	*Bothrops asper*	P-I	X-ray	Lingott et al. (2009)
Adamalysin II (1IAG)	*Crotalus adamanteus*	P-I	X-ray	Gomis-Rüth et al. (1993)
Flavoridin (1FVL)	*Protobothrops flavoviridis*	P-IIa (D-domain)	NMR	Senn and Klaus (1993)
H2-proteinase (1WN1)	*Protobothrops flavoviridis*	P-I	X-ray	Kumasaka et al. (1996)
Disintegrin (1Z1X)	*Echis carinatus*	P-IIa (D-domain)	X-ray	Hassan et al. (2005)

subclass (Leonardi et al., 2019). This protein is an apparent 21 kDa glycoprotein homologous to P-III SVMPs, lacking a metalloproteinase domain, thus consisting of only a partial disintegrin-like domain and an adjacent cysteine-rich domain (Leonardi et al., 2019). Fig. 3 represents the sub classification of the P-III class of SVMPs.

Previously in our laboratory, we reported the three dimensional structure of the protein bothropasin a P-III isolated from *Bothrops jararaca* venom (Muniz et al., 2008). Sequence alignment and comparative analysis with 41 other P-III SVMPs from NCBI revealed a significant pattern of sequence arrangement in the hyper-variable region (HVR) of members of this class (Muniz et al., 2008). A sub-group in the aligned sequences was highly homologous to bothropasin and showed a conserved sequence in the region of the so-called HVR, therefore referred to as highly conserved region – HCR. This conserved sequence was very distinct from the HVR of the sub-group that encompasses the protein VAP1, suggesting that this HVR vary from one group to the other, but it is conserved within those of bothropasin. Considering these data, we proposed a new classification for the P-III class into two sub-groups, the P-III-HCR and the P-III-HVR (Muniz et al., 2008). Additionally, we have reported the transcriptome analysis of bioactive agents from the venom gland of *Bothrops alternatus* (De Paula et al., 2014). Although consistent with a previous report from literature (Vonk et al., 2013), we identified copies of venom prolyl endopeptidases in the venom gland. It is noteworthy to mention the presence of P73 and P123 prolyl residues preceded by conserved K72 and H122 residues in members of the P-III class (De Paula et al., 2014). This data suggest the presence of two prolyl endopeptidase cleavage sites only in P-III members, thereby further distinguishing the P-III class from the P–I and P-II classes. Additionally, we hypothesized that the P-III SVMPs may be cognate substrates of the venom prolyl endopeptidases (De Paula et al., 2014).

The ECD (glutamic, cystein, aspatic) motif is a putative collagen interacting motif present in the disintegrin-like domain of most of the P-III class members (Fox and Serrano, 2008b; Markland and Swenson, 2013). Stejnihagin A and stejnihagin B are P-III representatives that were cloned from the venom of *Trimeresurus stejnegeri* (Wan et al., 2006b). Stejnihagin A has a SECD collagen interacting motif whereas stejnihagin B has a TECD collagen interacting motif. On the contrary, a few members of the P-III class contain the canonical RGD motif typical of the P-II class (Mazzi et al., 2007). Even though they contain a motif peculiar to the disintegrins, till date there is no available data that have tentatively excluded them from P-III SVMPs. An example is BjussuMP-I, a P-III hemorrhagic metalloproteinase from *Bothrops jararacussu* venom (Mazzi et al., 2007). The primary structure of this protein showed a conserved RGD-motif in the disintegrin-like domain. Activity reports showed that BjussuMP-I can induce lyses in fibrin clots and inhibit ADP-

and collagen-induced platelet aggregations (Mazzi et al., 2007).

Most members of the P-III class have a conserved stretch of six cysteinyl residues in their metalloproteinase domain as observed in stejnihagin A and B – Cys^{123}, Cys^{163}, Cys^{165}, Cys^{170}, Cys^{187} and Cys^{203} (Wan et al., 2006b). However, studies have identified the seventh cysteinyl residue in some SVMPs, which may be responsible for additional activity of the proteins (Fox and Serrano, 2005). Some studies have correlated the presence of the seventh cysteinyl residue to vascular apoptosis-inducing activity (Trummal et al., 2005; You et al., 2003). Additionally, this seventh cysteinyl residue may be conserved in the P-IIIb subclass since representatives of this subclass (including VAP1) have been demonstrated to potently induce apoptosis of vascular endothelial cells. Fig. 4 shows the amino acid sequence of bothropasin, a PIII isolated from *B.jararaca* venom, with emphasis on the disulfide bridges (Muniz et al., 2008). Table 3 shows the sub-classification of the P-III SVMPs and some examples of these proteins.

2. Domains functions

The resolution of the three-dimensional structures of SVMPs domains were important to the characterization and classification of these metalloproteinases as well as to elucidate the relationship between the domains and their specific functions during snake envenomation. Table 4 shows the SVMPs available in the Protein Data Bank (PDB), which 3D-structures were determined.

As previously described, studies have shown that some activities of SVMPs attributed to one of the domains are enhanced by the presence of another domain. In addition, we will provide a general description of the activities assigned to each domain in the following subsections.

2.1. Signal peptide and pro-domain

Unprocessed SVMPs contain a signal sequence that serves as an export signal and a molecular address label (Jia et al., 1996). Meanwhile the pro-domain contains a conserved stretch of PKMCGVT that controls maturation of SVMP by a cysteine switch mechanism (Ompraba et al., 2010; Takeda et al., 2012).

2.2. Metalloproteinase domain

The metalloproteinase domain of the SVMP classes represents the catalytically active domain of all the identified members. Three-dimensional structures of SVMPs revealed that this domain has an oblate ellipsoidal format with a small lower region and an upper main region containing the active site. Interesting, the C-terminal of the lower region is formed by a helix preceded by an irregular folded domain that is considered essential for substrate recognition (Takeda et al., 2012).

In the early part of this review, we have coalesced several reports that identified the presence of a zinc-binding scaffold in the M domain (Bernardes et al., 2013; Chou et al., 2013; Da Silva et al., 2012; Herrera et al., 2015; Komori et al., 2014; Lingott et al., 2009; Patiño et al., 2010). This identification validates that the biological activities of such domain solely depend on a zinc-aided catalysis. To further support this claim, researchers highlighted the presence of a unique HEXXHXXGXXH consensus sequence, which is responsible for coordinating the putative binding of zinc metal. The report that punctuated the loss of a metalloproteinase domain in the new P-IIIe subclass revealed its lack of metalloproteinase specific function/activities (Leonardi et al., 2019). Put together, the function of this domain depends on the pentahedrally coordinated zinc reinforced by the unique sequence of methionine called the “methionine turn” (Met-turn) (Markland and Swenson, 2013).

Recently, an immunological probe assay unveiled the presence of a conserved sequence of 21 amino acids (KARMYELANIVNEILRYLYMH) constituting a B-cell epitope in the M domain (Molina et al., 2018). This study hypothesized that the function of the M domain may be linked to immunological reaction involving migration of inflammatory cytokines and activation of various pathways underlining the pathological phenotype of snake envenomation (Molina et al., 2018). However, more data are necessary to validate this assumption.

One of the most studied functions mediated by the metalloproteinase domain during snake envenomation is its hemorrhagic activity, which is marked by hydrolysis of basement membrane proteins, such as laminin and type IV collagen that surround endothelial cells in the capillaries leading to local hemorrhage and in some cases causing a systemic effect (bleeding) (De Souza et al., 2016). Interestingly, Herrera et al. (2015) showed through an *ex vivo* model of mouse cremaster muscle tissue that P-II and P-III SVMPs co-localize with type IV collagen in capillaries and arterioles (microvessels) whereas P–I SVMPs has a widespread localization throughout the tissue. This difference in tissue localization is probably responsible for the high hemorrhagic activity of P-II and P-III members compared to the P–I class (Herrera et al., 2015). Moreover, the metalloproteinase domain is also important in non-hemorrhagic proteins, being associated with fibrino(geno)lytic and apoptosis inducing activities. In *in vitro* experiments, endothelial cell apoptosis is more pronounced after envenomation by non-hemorrhagic SVMPs (Baldo et al., 2008), and it occurs when the catalytic domain disrupts the molecular assembly formed between focal adhesions of endothelial cells and the extracellular matrix (Díaz et al., 2005). Such apoptotic mechanism may be underlined by alterations in the endothelial cell-integrin-extracellular matrix assembly resulted from metalloproteinase proteolytic activity. When such assembly is intact, the activities of certain proteins capable of triggering apoptosis remain suppressed.

Studies have also revealed the fibrino(geno)lytic activity of the metalloproteinase domain. There have been several reports of alteration in ligand-receptor interaction; such alteration corresponds to the ectodomain sheddase activities of this domain (Ho et al., 2002; Moura-da-Silva et al., 1996; Moura-da-Silva and Baldo, 2012). For instance, Oliveira et al. (2009) showed how atroxlysin-III from the Peruvian pit viper snake (*Bothrops oligolepis*) induced glycoprotein VI shedding, culminating into platelet dysfunction (Oliveira et al., 2019). Previously, the sheddase activity of atrolysin a, a P-III SVMP from *Crotalus atrox* venom, was demonstrated against collagen IV and annexin in cultured human fibroblasts (Pinto et al., 2007). Similar effects were suggested for jararhagin, a P-III SVMP from *Bothrops jararaca* upon TNF-α precursor (Moura-da-Silva et al., 1996).

2.3. Disintegrin and disintegrin-like domains

The disintegrin and the disintegrin-like domains have been briefly described at the early part of this review. We have also provided evidences from literature about the presence of an integrin-binding motif in these domains, its peculiarity and its variants. It is well-know that almost all the P-III SVMPs have a disintegrin-like domain that contains a D/ECD-conserved motif instead of the canonical RGD-motif of the disintegrins (P-II class).

The function of both disintegrin and disintegrin-like domains is marked by the presence of the conserved motif. For example, the disintegrin domain of P-II SVMPs is responsible for platelet aggregation inhibition due to its binding to $\alpha_{IIb}\beta_3$ integrin (Masuda et al., 2000; Ramos et al., 2008). Since integrins represent adhesive frameworks (although not exclusive); connecting the cellular cytoskeleton to extracellular matrix components, the interaction between disintegrins and integrins may also limit cell to cell and cell-ECM interactions. Interestingly, most of the activities of the disintegrins are marked by their interaction with integrins. Structurally, disintegrins can vary depending on the type of integrin-binding motif present in them and such variation may account for differences in target selection and substrate diversity (Muniz et al., 2008). Disintegrin-integrin interaction also depends on amino acid sequences present in the disintegrin loop (a structural feature that encloses the integrin binding motif), and in the C-terminus of the disintegrin. The presence of these two sequences culminated to

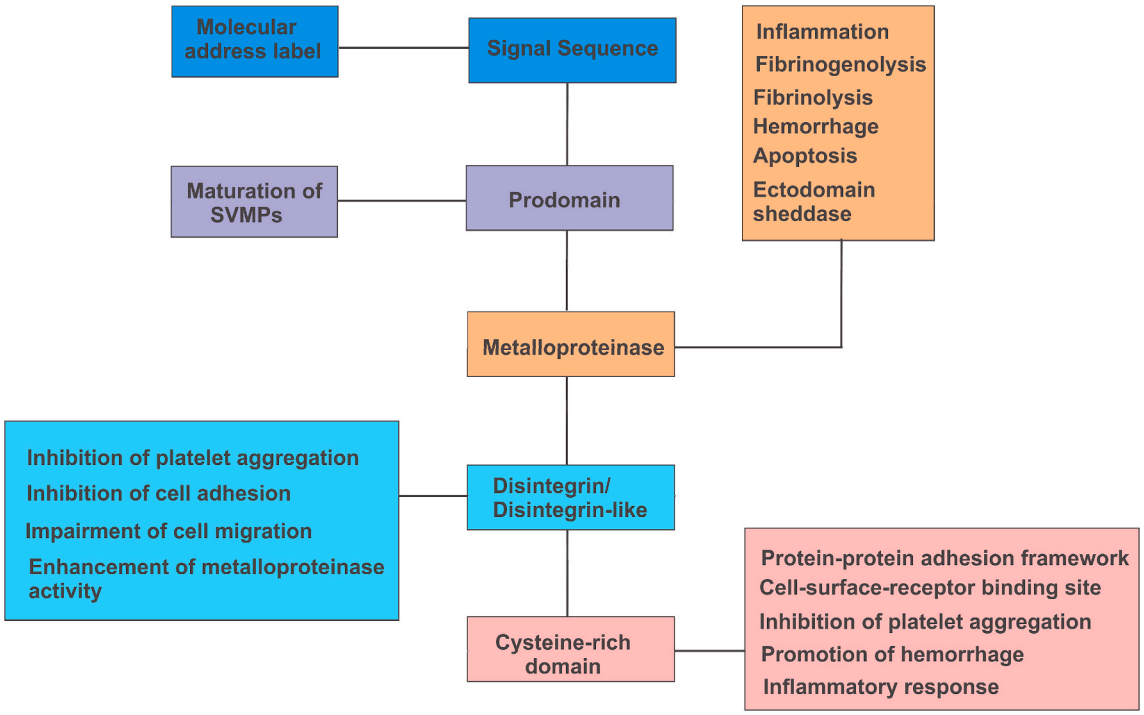

Fig. 5. Summary of SVMP domains and their functions.

variation in the integrin binding (Chang et al., 2017).

In general, disintegrins can inhibit adhesion and migration of several cell lines. Recently, Montealegre-Sanchez and co-workers reported that Lansbermin-l, a low molecular weight RGD-disintegrin from *Porthidium lansbergii lansbergii*, inhibited the adhesion and migration of MCF7 and MDA-DB 231 breast cancer cells by interfering with α_2 or β_1-containing integrins (Montealegre-Sánchez et al., 2019). Furthermore, Chalier et al. (2020) characterized the RGD-disintegrin Dabmaurin-1 isolated from *Daboia mauritanica* snake. Dabmaurin-1 inhibited HMEC proliferation, adhesion and migration to several ECM components, as well as tube formation after interaction with β_1-containing integrins (Chalier et al., 2020). Additionally, in our laboratory, we demonstrated that Dis*Ba*-01, a RGD-disintegrin, inhibited cell migration and adhesion in murine breast tumor cell line 4T1BM2. Our data confirmed that Dis*Ba*-01 hampered cell cycle progression at S phase and induced autophagy (Lino et al., 2019). In addition, we demonstrated that the blockade of $\alpha_v\beta_3$ integrin in oral squamous carcinoma cells by Dis*Ba*-01 inhibits cell directionality of migration (Montenegro et al., 2017).

The presence of the non-catalytic disintegrin-like and cysteine-rich domains is strongly responsible for the hemorrhagic activity of the P-III class members (Escalante et al., 2011). Escalante and co-workers revealed that the activities of P-III SVMPs are not limited to their catalytic domain and that most of the activities may be in tandem or cumulatively mediated by more than one domain. This means that P-III SVMPs containing an intact metalloproteinase domain and a disintegrin-like domain may be more potent in their activities than those lacking the non-catalytic domain (Escalante et al., 2011). The ECD-containing disintegrin-like proteins of the P-III class have also been shown to inhibit the adhesion of cells to collagen (De Luca et al., 1995; Jia et al., 2000; Souza et al., 2000; Suntravat et al., 2016; Tanjoni et al., 2010; Zhou et al., 1996).

It is important to highlight that 3D-structures of P-III SVMPs showed that the metalloproteinase domain alongside with the disintegrin-like and the cysteine-rich domains (MDC) form a C-shaped structure that places the HVR region of the cysteine-rich domain near to the catalytic site of the metalloproteinase domain. This is important to substrate recognition, because this C-shaped configuration allows a flexibility between the catalytic site and the exosite of P-III throughout the catalytic cycle (Takeda, 2016).

2.4. Cysteine-rich domain

Structural studies have shown that the cysteine-rich domain (C-domain) of bothropasin is formed by two α-helices, 4 β-strands and loops (Muniz et al., 2008; Takeda et al., 2006). The loop is stabilized by the presence of a disulfide bond found in the C-domain, which contain the hyper-variable region (HVR) or the highly-conserved region (HCR), depending on the specific subclass of P-III SVMPs (Muniz et al., 2008). Structural studies indicated that the C-domain could bind to a free- or membrane-bound target protein positioning the M domain to its substrate for proteolytic attack (Takeda et al., 2006). In addition, the HVR can have an essential role in protein-protein interactions, suggesting a structural correlation for the variety of biological activities found in the P-III class (Takeda, 2016). As mentioned before, the HVR localizes opposite to the catalytic site in the C-shaped MDC structure, thus it has been addressed with the function of substrate/peptide recognition (Takeda, 2016; Takeda et al., 2012). For instance, Menezes et al. (2008) showed that the HVR of the C-domain formed a protein-to-protein adhesive framework capable of inducing leucocyte rolling in microcirculation (Menezes et al., 2008). In another study, they demonstrated through site directed mutagenesis that the HVR of this domain inhibited platelet aggregation (Menezes et al., 2011). Additionally, Serrano et al. (2007) showed that the von Willebrand factor-mediated platelet aggregation was inhibited by the C-domain (Serrano et al., 2007). Solid-phase binding assay using type I collagen and vWF revealed that this domain contains exosites that can act as a cell-surface-receptor-binding site (Serrano et al., 2005), thereby revealing the role of the C-domain in the promotion of hemorrhage through substrate recognition, target selection and positioning of the metalloproteinase domain for its proteolytic activity (Serrano et al., 2006).

The C-domain also plays a critical role in inflammation. In functional association with the disintegrin-like domain, it has been shown to sufficiently cause leucocyte rolling and release of pro-inflammatory

cytokines in the early stage of acute inflammatory response (Clissa et al., 2006). However, a recent report showed that the catalytic metalloproteinase domain of SVMPs is the principal effector of leucocyte recruitment, suggesting that the C-domain could also present an inflammatory response only in the absence of the M domain (Zychar et al., 2020). Fig. 5 shows the summary of the SVMP domains and its functions.

3. Concluding remark

Snake Venom Metalloproteinases are diverse in both structure and activities. The most recent classification recognizes three classes: P–I, P-II and P-III, including sub-classification of P-II and P-III classes. Furthermore, advancement in protein characterization and sophistication in molecular techniques continue to avail scientists the opportunity to identify novel SVMPs and correlate activities to specific domains. However, the exact sub-domain structures responsible for these activities have only been speculated in some cases. In addition, despite the identification of novel SVMPs, not all have been properly classified, especially into the P-II and P-III sub-classes. Therefore, as the field of molecular toxinology advances, scientists should maximize the use of available tools to characterize and identify the exact sub-domain structures that are responsible for their specific activities; as this will not only permit proper classification, but also expand the current understanding on the contribution of SVMPs to snake envenomation. Snake genomics, transcriptomics and proteomics contribute qualitatively and quantitatively to studies of structure-function relationship of venom components, adding new information that ultimately help to characterize toxins, notably SVMPs. As the Venomics develops, our knowledge evolves, opening doors to new discoveries in the field.

Author contributions

Original Draft Preparation, O.T.O. and D.H.F.S.; Writing - review & editing, O.T.O., D.H.F.S., P.K.S., H.S.S; Funding acquisition D.H.F.S. and H.S.S. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

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