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Review

Snake venom proteases affecting hemostasis and thrombosis

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Abstract

The structure and function of snake venom proteases are briefly reviewed by putting the focus on their effects on hemostasis and thrombosis and comparing with their mammalian counterparts. Up to date, more than 150 different proteases have been isolated and about one third of them structurally characterized. Those proteases are classified into serine proteases and metalloproteinases. A number of the serine proteases show fibrin(ogen)olytic (thrombin-like) activities, which are not susceptible to hirudin or heparin and perhaps to most endogenous serine protease inhibitors, and form abnormal fibrin clots. Some of them have kininogenase (kallikrein-like) activity releasing hypotensive bradykinin. A few venom serine proteases specifically activate coagulation factor V, protein C, plasminogen or platelets. The venom metalloproteinases, belonging to the metzincin family, generally show fibrin(ogen)olytic and extracellular matrix-degrading (hemorrhagic) activities. A few venom metalloproteinases show a unique substrate specificity toward coagulation factor X, platelet membrane receptors or von Willebrand factor. A number of the metalloproteinases have chimeric structures composed of several domains such as proteinase, disintegrin-like, Cys-rich and lectin-like domains. The disintegrin-like domain seems to facilitate the action of those metalloproteinases by interacting with platelet receptors. A more detailed analysis of snake venom proteases should find their usefulness for the medical and pharmacological applications in the field of thrombosis and hemostasis. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Snake venom; Serine protease; Metalloproteinase; Metzincin; Hemostasis; Thrombosis

1. Introduction

At present, there are more than 200 species of venomous snakes on the earth. They are classified into four major families: (1) *Hydrophiae*, (2) *Elapidae*, (3) *Viperidae* and (4) *Crotalidae*. To provide victims with various damages, they have a venom gland that synthesizes, stores and secretes about 50–60 protein/peptide components with different structures and functions, as either the active or the inactive precursor form, into the site of their bite. The precursor forms of components are activated by a special mechanism after the secretion. The venom components seem to be fairly common and similar to one another within each family of snakes (e.g., nerve toxins are generally found in the *Hydrophidae* and *Elapidae* venoms and hemorrhagic and myonecrotic toxins are generally found in the venoms of the other two families of snakes), but they are basically different depending on each snake species [1,2]. There are several unique components that have been so far isolated from the venom of only one snake species. Recently Daltry et al. [3] have published an interesting report that venom components have a considerable geographical variation even within each snake species in close association with its diet, in accord

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with the previous observation that venom components even from the same snake species happens to be quite different depending on a batch of the venom.

These venom components include neurotoxins, cytotoxins, cardiotoxins, nerve growth factors, lectins, von Willebrand factor (VWF)-binding proteins, factor IX/X-binding protein, platelet glycoprotein (GP) Ib-binding proteins, disintegrins, bradykinin-potentiating peptides (angiotensin-converting enzyme inhibitors), natriuretic peptides, various enzymes (e.g., proteases, phospholipases, phosphodiesterases, choline esterases, aminotransferases, L-amino acid oxidase, catalase, ATPase, hyaluronidase, NAD nucleosidase, and β-glucosaminidase) and enzyme inhibitors [1,2].

Since proteases are present in most of the venoms except for the *Hydrophiae* venoms, they have been studied by many investigators since the beginning of this century when the blood clotting by a snake venom was first recognized, although the first venom protease with a clotting activity was purified in the 1950s. Proteases so far isolated are generally classified by the structure into (1) serine proteases and (2) metalloproteinases. There is only a weak or indirect evidence for the presence of thiol proteases and aspartic proteases in the venoms. Some of them seem to degrade mammalian tissue proteins in the site of bites in a nonspecific manner to immobilize the victims. A number of them, however, cleave some of plasma proteins of the victims in a relatively specific manner to give potent effects, as either the activators or the inhibitors, on their hemostasis and thrombosis, such as blood coagulation, fibrinolysis and platelet aggregation [1,2,4–8].

The purpose of this short review is to discuss the literature and the authors' own data recently published on the biochemical, enzymatic and structural properties of those snake venom proteases affecting hemostasis and thrombosis.

2. Snake venom proteases

According to the research [4–8] on an inventory of snake venom proteases, more than 150 different proteases have been so far purified, either completely or partially, and functionally characterized since the 1950s. The complete amino acid sequences of about 40 out of those proteases have been determined by protein sequencing or deduced from the nucleotide sequence of the cDNA. Recently, the three-dimensional (3D) structures of five venom proteases, four metalloproteinases [9–12] and one serine protease [13], have been determined by X-ray crystallographic analysis and have made it possible to understand their structure–function relationships in more detail.

The venom proteases so far structurally characterized are either serine proteases or metalloproteinases with fibrin(ogen)olytic activity with a few exceptions. It is not unusual to find one venom containing both fibrinolytic (anticoagulant) and fibrinogenolytic (coagulant) activities or one protease showing both activities. Such a venom or protease may exert anticoagulant or coagulant effects depending on the concentration used.

3. Serine proteases

Some of the serine proteases have both fibrinogenolytic and fibrinolytic activities, but a number of them have only fibrinogenolytic activity and are also called 'thrombin-like' proteases if they show 'fibrinogen clotting' activity [4–8]. However, their actions toward fibrinogen as well as the other substrates of thrombin are not exactly identical to those of thrombin. Instead of fibrin(ogen)olytic activity, several venom serine proteases have the activity for releasing bradykinin from kininogen like mammalian kallikrein (or kininogenase) [14,15] and are also called 'kallikrein-like' proteases [15]. In addition, there have been a few reports on the venom serine proteases with a unique activity, such as the activation of factor V [16], protein C [17], plasminogen [18,19] or platelets [20].

3.1. Thrombin-like serine proteases

Mammalian α-thrombin (an activated form of prothrombin (blood coagulation factor II)) is a multifunctional serine protease. The previous extensive studies both on the function and the structure by many investigators have revealed that thrombin plays various important roles in hemostasis and thrombosis in mammals. Since its major role is to

Table 1
Venom serine proteases with the complete amino acid sequence

Common name (species source)	Substrate(s)	Ref.
Thrombin-like		
Ancrod (*Calloselasma rhodostoma*, formerly *Agkistrodon rhodostoma*)	Fibrinogen (A,[B])[a], factor XIII	[23,24]
Batroxobin (*Bothrops atrox moojeni*)	Fibrinogen (A,[B]), factor XIII?	[25]
Bilineobin (*A. binlineatus*)	Fibrinogen (B,[A])	[26]
Bothrombin (*B. jararaca*)	Fibrinogen (A), factor VIII?	[27]
Calobin (*Crotalus atrox*)	Fibrinogen (A,[B])	[28]
Crotalase (*C. adamanteus*)	Fibrinogen (A,[B])	[29]
Flavoxobin (*Trimeresurus flavoviridis*)	Fibrinogen (A)	[30]
Gyroxin analogue (*Lachesis muta muta*)	Fibrinogen (A,[B])	[31]
TM-VIG (*T. mucrosquamatas*)	Fibrinogen (B)	[32]
Kallikrein-like		
Halystase (*A. halys blomhoffii*)	Kininogen, fibrinogen[b]	[33]
Gilatoxin (*Heloderma horridum horridum*)	Kininogen	[34]
Others		
ACC-C (*A. contortrix contortrix*)	Protein C	[17]
PA-BJ (*B. jararaca*)	Platelets	[20]
RVV-V (*Vipera russelli*)	Factor V	[16]
TSV-PA (*T. stejnegeri*)	Plasminogen	[18,19]

[a]Released fibrinopeptides are shown in parentheses. The fibrinopeptide in brackets is released much slower than the other.
[b]Fibrinogen is degraded without releasing either fibrinopeptide A or B (see the text).

convert fibrinogen (factor I) to fibrin clots, which is then cross-linked by activated factor XIII, by releasing fibrinopeptides A and B from the Aα and Bβ chains of fibrinogen, respectively, by limited proteolysis, it is also called fibrinogenase. However, in addition to the action on fibrinogen, thrombin is also involved in the stimulation of blood coagulation by activating factor V, VIII and XIII (and possibly factor VII and XI). Its complex with thrombomodulin in endothelial cells in turn activates protein C to inhibit blood coagulation by inactivating the activated forms of factor V and VIII. Furthermore, it also stimulates fibrinolysis and activates platelet aggregation [21,22].

Table 1 lists most of the venom serine proteases of the known complete amino acid sequences (Fig. 1). There are many more serine proteases that have been isolated from the venoms, but not yet structurally characterized. Some venoms contain more than one serine proteases with similar or different activities, generating some confusion. For example, the two complete amino acid sequences of ancrod with 62 different residues have been published [23,24]. The first sequence has been determined by direct protein sequencing of a thrombin-like enzyme isolated from the venom of *Calloselasma rhodostoma*, formerly called *Agkistrodon rhodostoma* [23], and the second sequence has been deduced from the nucleotide sequence of the cDNA perhaps encoding a similar enzyme from the same venom [24], raising a question whether the second sequence is for ancrod or another thrombin-like serine protease in the same venom. Another problem is the terminological confusion. For example, an extract with the fibrinogen clotting activity from the venom of *Bothrops jararaca* was first termed ‘reptilase’ in 1957 [35]. This term, however, has been used incorrectly afterwards in many literatures as the trivial name synonymous with ‘batroxobin’ from the venom of *B. atrox moojeni*. To avoid this sort of terminological problem, we gave a new term ‘bothrombin’ to a thrombin-like serine protease that had been isolated from the venom of *B. jararaca* and structurally characterized, because we were unaware of the identity between ‘reptilase’ and ‘bothrombin’ without knowing the amino acid sequence of reptilase first recognized in the venom of *B. jararaca* [27].

Since all of the serine proteases listed in Table 1

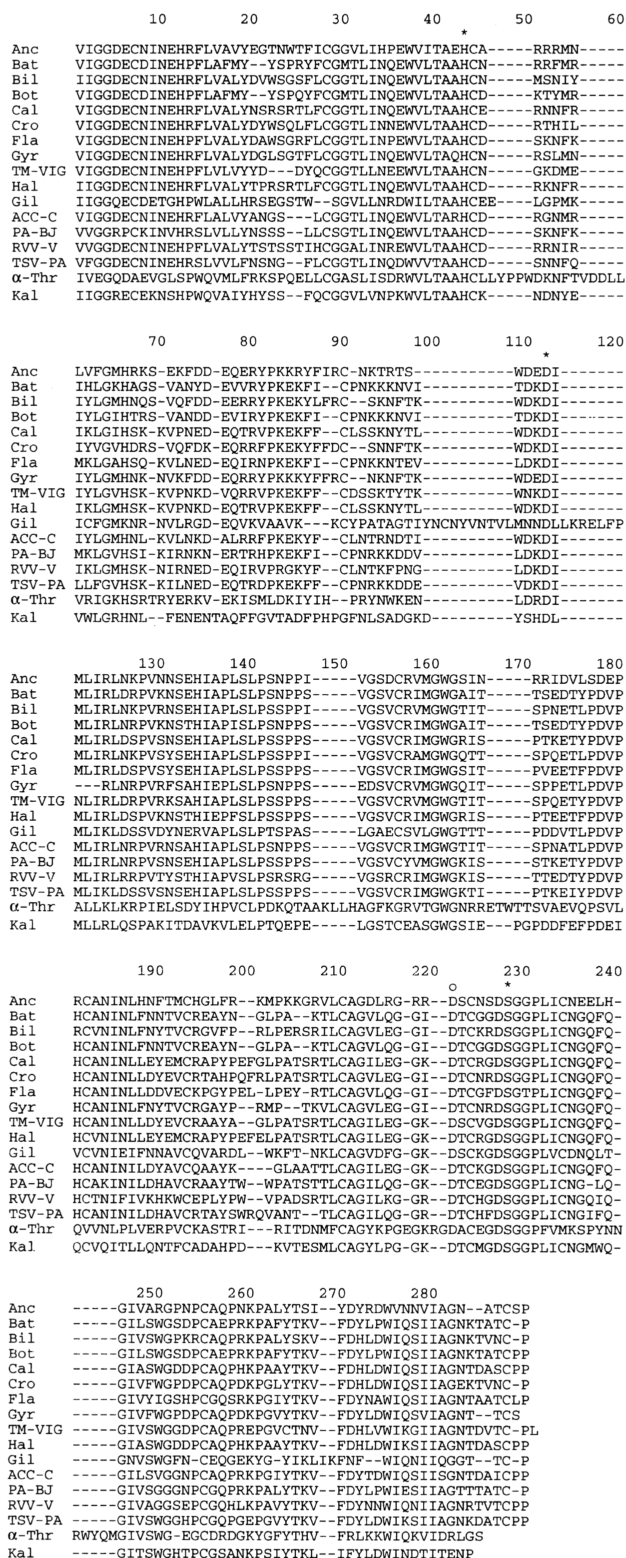

Fig. 1. Comparison of the amino acid sequences of snake (or lizard) venom and mammalian serine proteases. Anc, ancrod [23]; Bat, batroxobin [25]; Bil, bilineobin [26]; Bot, bothrombin [27]; Cal, calobin [28]; Cro, crotalase [29]; Fla, flavoxobin [30]; Gyr, gyroxin analogue [31]; TM-VIG [32]; Hal, halystase [33]; Gil, gilatoxin (lizard) [34]; ACC-C [17]; PA-BJ [20]; RVV-V [16]; TSV-PA [18]; α-Thr, bovine α-thrombin [36]; Kal, porcine glandular kallikrein [37]. Gaps have been inserted to maximize similarities. Tentative catalytic triad residues and the major substrate-binding site are indicated by * and ○, respectively.
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have His, Asp and Ser residues in the corresponding positions to the catalytic triad established in mammalian serine proteases (Fig. 1) and are inhibited by common serine protease inhibitors such as diisopropyl fluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF), it is obvious that they are serine proteases. All of these serine proteases are likely to be glycoproteins each containing a few Asn (N)-linked glycosylation sites in non-homologous positions to one another in the sequence. Cys residues in their molecules are all disulfide-linked, and their pairing is identical to that of mammalian trypsin [25,26].

As shown in Table 1, most of the venom thrombin-like serine proteases preferentially release either fibrinopeptide A or B from fibrinogen to produce abnormal fibrin clots composed of short polymers that are rapidly dispersed and no longer cross-linked by activated factor XIII, resulting in the disruption of the blood coagulation system of victims. This implies that many or most of 11 basic amino acid residues implicated in the fibrinogen recognition exocites 1 and 2 near the active site cleft of thrombin and probably involved in binding to hirudin and heparin are not homologously present in the venom thrombin-like serine proteases, in accord with their insensitivity to hirudin (a thrombin-specific inhibitor) and in most cases to SERPINs (endogenous serine protease inhibitors) such as antithrombin-III (AT-III). However, the possibility remains that their fibrinogen recognition may depend on some basically different mechanism of interaction.

In addition to their action toward fibrinogen different from that of thrombin, none of the venom thrombin-like serine proteases shows all the multifunctions of thrombin, although some of them also activate factor VIII and/or XIII or aggregate plate-

lets like thrombin. In our recent study [27], bothrombin releases only fibrinopeptide A from fibrinogen, but in a longer incubation, it cleaves the Bβ chain at Arg42 (T. Matsui, unpublished data). In addition, it activates factor VIII with a much lower specific activity than that of thrombin, and also induces platelet aggregation, but without releasing serotonin and only in the presence of exogenous fibrinogen in a manner entirely different from that of thrombin.

3.2. Kallikrein-like serine proteases

The second group of the venom serine proteases are kallikrein-like enzymes and release hypotensive bradykinin from kininogen in mammalian plasma. Recently, we have isolated a kallikrein-like serine protease from the *A. halys blomhoffii* venom and termed halystase [33]. Interestingly, the enzyme has higher sequence similarities to kallikrein (42% identity) than thrombin (26%), although it has also high similarities to the venom thrombin-like serine proteases (66–72%) and shows some fibrinogenolytic activity. It cleaves the Bβ chain at Arg42 and slowly degrades the Aα chain of fibrinogen to generate a product that is no longer converted to normal fibrin clots by thrombin, resulting in the inhibition of normal fibrinogen clotting. Therefore, this serine protease induces the reduction of blood pressure as well as the inhibition of blood coagulation in the victims. The presence of another kallikrein-like serine protease with a potent activity but with different physicochemical properties from those of halystase has been recognized in the same venom, and a number of the other kallikrein-like serine proteases have been isolated from the venoms of *A. caliginosus*, *C. atrox* and *C. viridis*, but not yet structurally characterized [14,15]. Recently, a serine protease termed gilatoxin with kallikrein-like activity has been isolated from the venomous lizard (*Heloderma horridum horridum*) and structurally characterized as shown in Table 1 and Fig. 1 [34].

3.3. Other unique serine proteases

As shown in Table 1, some other venom proteases with a unique activity have been isolated and structurally characterized as serine proteases. These include RVV-V (factor V-activating enzyme) from the *V. russelli* venom [16], ACC-C (protein C activator) from the *A. contortrix* venom [13], PA-BJ (platelet aggregating enzyme) from the *B. jararaca* venom [20], and TSV-PA (plasminogen activator) from the *T. stejnegeri* venom [18,19]. Interestingly, TSV-PA cleaves the same peptide bond in plasminogen as mammalian plasminogen activators, but it lacks the kringle and finger domains that provide the fibrin-binding specificity.

4. Metalloproteinases

The venoms contain a variety of metalloproteinases that are highly toxic, resulting in a severe bleeding by interfering with the blood coagulation and hemostatic plug formation or by degrading the basement membrane or extracellular matrix components of the victims [38–40]. More than 100 metalloproteinases, including the isozymes from the same species, have been isolated and the amino acid sequences of about 20 enzymes have been determined. They are all zinc (Zn^{2+}) metalloproteinases with a Zn^{2+}-binding motif of HEXXHXXGXXH and belong to the metzincin family as well as matrixins such as mammalian matrix metalloproteinases, astacins such as crayfish collagenolytic protease and serralysins such as bacterial metalloproteinases [41]. Chelation of the Zn^{2+} ion with EDTA or 1,10-phenanthroline deprives them of their proteolytic and hemorrhagic activities.

4.1. Substrate specificities of metalloproteinases

Most of the venom metalloproteinases, either hemorrhagic or non-hemorrhagic, are fibrin(ogen)olytic enzymes as well as the serine proteases, cleaving preferentially the Aα-chain and slowly the Bβ-chain of fibrinogen [42]. Fibrolase from *A. contortrix contortrix* [43], atroxase from *C. atrox* [44] and lebetase from *V. lebetina* [45] degrade the α-chain of fibrin and fibrinogen faster than the β-chain. There is no report on venom fibrin(ogen)olytic metalloproteinase that specifically cleaves the γ-chain of fibrin(ogen) except for atrolysin F (Ht-f) from *C. atrox* [46]. Like the serine proteases, the metalloproteinases with the fibrin(ogen)olytic activity are also applicable for a medical treatment that lowers the fibrinogen

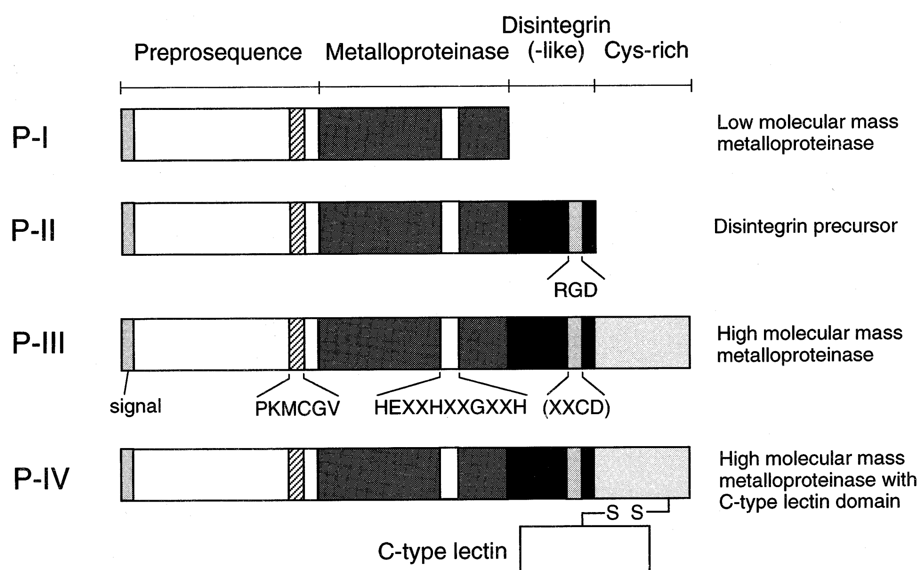


Fig. 2. Schematic structures of snake venom metalloproteinases. Snake venom metalloproteinases are classified into four groups by the domain structures [38,39]. Preprosequence deduced from the cDNA containing the signal peptide (18 residues) and the prosequence (about 170 residues) with a cysteine-switch motif (PKMCGV) is removed by the signal peptidase and autolytically or by the action of another unknown protease. The metalloproteinase domain (about 200 residues) has the Zn^{2+}-binding motif (HEXXHXXGXXH). The disintegrin-like domain has a XXCD sequence instead of the RGD sequence in disintegrins. Class P-I consists of low molecular mass metalloproteinases such as HR2a, HT-2, atrolysin (Ht) B, C, E, LHF-II and trimerelysin II (H_2-proteinase). Class P-II consists of precursor proteins of disintegrins (trigramin, rhodostomin, etc.) and atrolysin E. The disintegrin domain might be released autolytically or by the action of another unknown protease. Class P-III consists of high molecular mass metalloproteinases such as trimerelysin I (HR1B), atrolysin (Ht) A and jararhagin composed of metalloproteinase, disintegrin-like and cysteine-rich domains. Class P-IV consists of high molecular mass metalloproteinases containing an additional disulfide-linked C-type lectin-like domain such as russellysin (RVV-X), carinactivase-1 and mucrotoxin A, although the preprosequence is still hypothetical since the cDNA has not yet been characterized.

level in plasma or solubilizes the coagulated plasma (thrombolysis).

Substrate specificities of metalloproteinase have been assayed with casein, insulin B peptides and intermolecularly quenched fluorogenic peptide substrates [38,39,47]. The hemorrhagic metalloproteinases trimerelysin I (HR1A), HR2a and non-hemorrhagic trimerelysin II (H_2-proteinase) from *T. flavoviridis* as well as Cbfib2 (*C. basiliscus*) preferentially cleave at the peptidyl bond of Pro 516 and Met 517 in the Aα-chain of fibrinogen. These enzymes generally have the primary specificity toward substrates of more than pentapeptides with a hydrophobic and bulky P1′ residue such as Leu and Met [38]. Factor X activator (russellysin or RVV-X) from Russell's viper (*V. russelli*) venom [48], which also activates factor IX, seems to require Arg residue at the P1 site [47]. Hemorrhagic atrolysins (Hts) from *C. artox* degrade basement membrane proteins, including type IV collagen, nidogen, laminin and fibronectin. The initial cleavage site of type IV collagen by atrolysin E (Ht-e) has been identified to be the Ala258–Gln259 bond located in a pepsin-susceptible triplet interruption region of the α1(IV) chain and Gly191–Leu192 bond located in a triple-helical region of the α2(IV) chain [39,49]. The metalloproteinase atrolysin C (Ht-d) cleaves the cartilage aggrecan core protein at two sites of Asn341–Phe342 and Glu373–Ala374 bonds [50].

Hemorrhagic and fibrin(ogen)olytic metalloproteinases have been mainly purified from the crotalid and viperid venoms, but recently, some interesting homologues have been also isolated from the elapid venom. Mocarhagin, a metalloproteinase purified from the cobra venom of *Naja mocambique mocambique*, preferentially cleaves the Glu282–Asp283 bond of platelet GPIbα that plays a key role in the platelet aggregation by interacting with plasma VWF, and also the Tyr10–Asp11 bond of neutrophil P-selectin glycoprotein ligand-1 that mediates neutrophil roll-

ing at the site of inflammation [51]. Those cleavage sites are within a negatively charged amino acid sequence beginning with Asp residue and ending in close proximity to sulfated Tyr residue(s). Mocarhagin can also cleave human fibrinogen Aα-chain at Lys413–Leu414 and Phe501–Asp502 bonds in a long incubation. It also shows a lectin-like activity, as assayed by hemagglutination, that can be inhibited with sulfated polysaccharides but not with EDTA [52]. The mocarhagin-like metalloproteinases cleaving GP Ibα seem to be widely distributed in the viperid venoms.

We have recently purified another unique metalloproteinase, kaouthiagin, from the cobra venom of *N. kaouthia* that specifically binds and cleaves human VWF at a single peptide bond between Pro708 and Asp709 to diminish the multimeric structures of VWF, resulting in loss of both the ristocetin-induced platelet aggregating and collagen-binding activities of VWF [53]. This cleavage site of VWF is located within a cluster of negatively charged *O*-glycosides. Kaouthiagin cleaves neither fibrinogen nor synthetic peptide substrates for trimerelysins. These elapid metalloproteinases seem to cleave the amino-terminal side of Asp residue in a region containing many negatively charged residues.

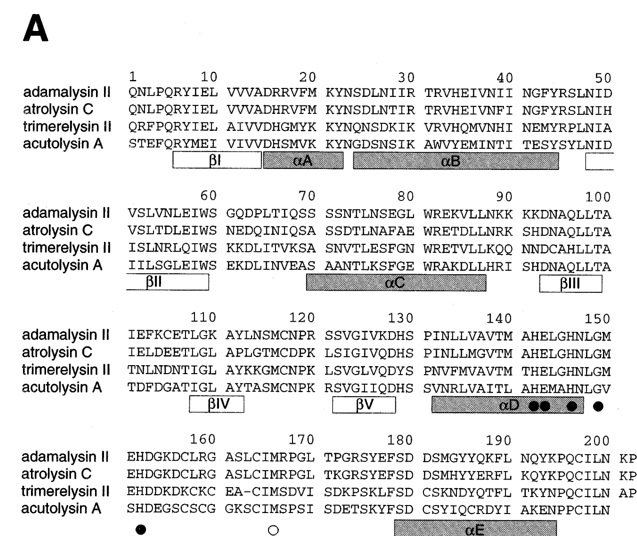
Jararhagin from the venom of *B. jararaca* has been shown to degrade platelet collagen receptor α2β1 integrin (GP Ia/IIa) in addition to fibrinogen and VWF, resulting in the inhibition of platelet aggregation [54]. Ivaska et al. [55] have shown that the artificial cyclic peptide, (C)TRKKHDNAQ(C), where the N- and C-terminal Cys forms the disulfide bond, designed on the basis of residues 90–98 of jararhagin in the proteinase domain inhibits the interaction between the integrin α2 subunit and collagen. Although a number of the metalloproteinases are α-fibrinogenase and inhibit platelet aggregation, it is possible that they interact with or degrade the receptors on platelets (integrins) essential for the aggregation either by the proteinase or the disintegrin-like domain (see next section).

4.2. Structures of metalloproteinases

The venom metalloproteinases are classified into four major groups by their domain structures of proteins or their cDNAs [38,39,56] (Fig. 2). Low molecular mass hemorrhagic or non-hemorrhagic metalloproteinases (class P-1), such as HR2a, HR2b and trimerelysin II (H_2-proteinase) from *T. flavoviridis*, ruberlysin (HT-2) from *C. ruber ruber*, atrolysin C (Ht-d) and E (Ht-e) from *C. atrox*, adamalysin II from *C. adamanteus* and LHF-II from *Lachesis muta muta*, consist of only the proteinase domain of about 25 kDa.

High molecular mass hemorrhagic metalloproteinases, such as trimerelysin I (HR1B) from *T. flavoviridis*, atrolysin A (Ht-a) from *C. atrox* and jararhagin from *B. jararaca*, contain the disintegrin-like and Cys-rich domains following the proteinase domain (class P-III). Disintegrins such as trigramin and rhodostomin are venom peptides of about 50–60 residues containing the RGD sequence that strongly inhibit platelet aggregation by interacting with platelet αIIbβ3 integrin (GP IIb/IIIa) [57]. It has been proposed that these disintegrins are generated by proteolytic processing of the precursor protein composed of the proteinase and disintegrin domains (class P-II). Instead of the RGD-containing disintegrin domain, class P-III metalloproteinases have the disintegrin-like domain containing a disulfide-bonded XXCD (mostly SECD) sequence in place of the RGD sequence. We have isolated jararhagin C from the venom of *B. jararaca* that has the identical sequence to the disintegrin-like domain of jararhagin and inhibits collagen- or ADP-induced platelet aggregation [58]. Recently, a recombinant protein composed of the disintegrin-like and Cys-rich domains of atrolysin A (Ht-a) has been shown to inhibit collagen- and ADP-stimulated platelet aggregation in a similar manner to disintegrins [59]. These findings suggest that the disintegrin-like domain also interact with integrin molecules or other receptors. Class P-III metalloproteinases are generally more hemorrhagic than those of class P-I, suggesting that the disintegrin-like domains play a key role in facilitating the action of these metalloproteinases by affecting platelet aggregation.

Another class of the venom metalloproteinases have an additional disulfide-linked C-type lectin domain (class P-IV). RVV-X [60] and carinactivase-1, the prothrombin activator from *Echis carinatus* [61], are members of this class, although none of their entire cDNAs has yet been characterized. The chimeric structures of the venom metalloproteinases (es-



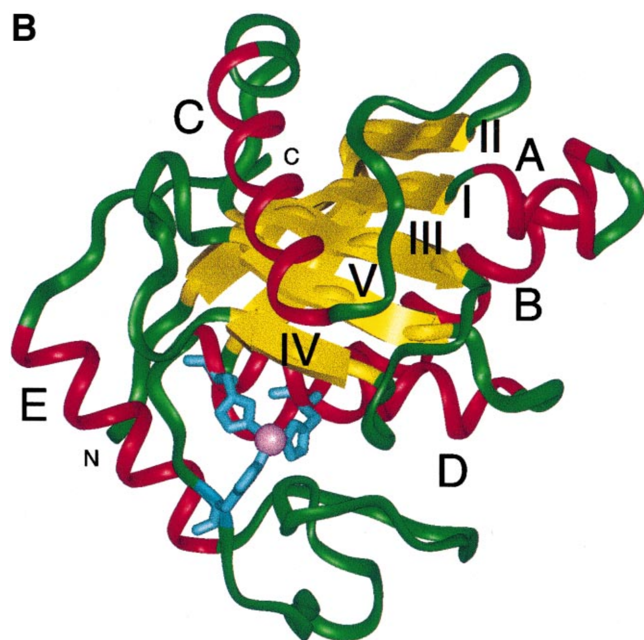


Fig. 3. Amino acid sequences of four snake venom metalloproteinases and the crystal structure of trimerelysin II (H_2 -proteinase). (A) Comparison of the amino acid sequences of adamalysin II (*C. adamanteus*), atrolysin C (*C. atrox*), trymerelysin II (*T. flavoviridis*) and acutolysin A (*A. acutus*). The α-helices and β-sheets from adamalysin II are expressed by hatched and white bars, respectively. The metzincin consensus sequence for the Zn^{2+}-binding motif (●) and the Met-turn residue (○) are shown. (B) The crystal structure of H_2-proteinase is shown as a ribbon model. The model was rendered on an IRIS Indigo 2 workstation using the Insight II (molecular simulations) and SYBYL/Base software packages. It is composed of two domains that are separated by a cleft occupied by the catalytic center consisting of Zn^{2+} ion and its chelators. The N-terminal region has the central core domain composed of five β-sheets (yellow) and four α-helices (red), whereas the C-terminal region has one helix and an irregularly folded portion [11]. N and C denote the N- and C-terminus of the polypeptide, respectively.
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pecially those containing the disintegrin-like domain) have been identified in mammalian membrane-anchored reproductive proteins (fertilins), myogenetic proteins (meltrins) and TNFα convertase (TACE), suggesting that they are members of the widely distributed basic protein family so called MDC, ADAMS or reprolysins [39,62].

These metalloproteinases are synthesized and stored in the venom gland as the inactive zymogens with a conserved thiol group in the prosequence PKMCGV blocking the active site by binding to Zn^{2+} ion. After the secretion from the gland, the proteolytic processing converts the zymogen to the active enzyme by removing this thiol group. This Cys-switch type activation mechanism, like that for mammalian matrix metalloproteinases, has been proposed for the venom metalloproteinases [56,63,64].

The 3D structures have been elucidated by X-ray crystallographic analysis with four venom metalloproteinases, adamalysin II from the *C. adamanteus* venom [9], atrolysin C [10], trimerelysin II [11] and acutolysin A from the *A. acutus* [12] (Fig. 3A). These results have revealed that they are ellipsoidal molecules with the shallow active site cleft separating a relatively irregular folded lower subdomain from the upper major domain composed of five stranded β-sheets and four α-helices (Fig. 3B). The catalytic Zn^{2+} ion in the active-site cleft is surrounded by three His residues (142, 146 and 152) and a water molecule is anchored to Glu143 in a tetrahedral manner. The substrate binding site is bordered by a Met turn (Met166), which forms a 'basement', and two 'walls' formed by a strand (residues 168–172) and an antiparallel β-strand of the upper main domain (residues 108–112). In general, the overall structure of the venom metalloproteinases appears to be similar to that of the astacin family, but distinct from that of the thermolysin family.

5. Perspectives

The recent advance in the studies on the venom

proteases affecting mammalian hemostasis and thrombosis are briefly reviewed in this article. The interesting results that the venom fibrin(ogen)olytic proteases have the activity to degrade fibrin clots or to produce abnormal fibrin clots of short polymers that are rapidly dispersed and perhaps lack the susceptibility to SERPINs have generated interest in their potential therapeutic application for the treatment of patients with occlusive arterial or venous thrombotic diseases without stimulating the endogenous fibrinolysis system [2,5,42,65].

The elucidation of the interacting and signal-transducing mechanisms through the integrins on platelets and the disintegrin-like domains of highly hemorrhagic metalloproteinases should bring some insights into the regulation of platelet aggregation. Synergistic effects of the disintegrin-like domain on the hemorrhage by the venom metalloproteinases suggest the presence of novel integrin molecules on platelets. Substrate specificities of the venom metalloproteinases toward VWF, aggrecan (ECM) and platelet receptors similar to those observed in proteolysis in vivo suggest that endogenous metalloproteinases belonging to the metzincin family may be present in mammalian plasma and participate in the degradation control of those proteins. Recently, the presence of an endogenous VWF-cleaving metalloproteinase has been recognized in human plasma, although it has not yet been completely purified and characterized [66,67]. It cleaves a single Tyr842–Met843 bond of VWF to produce a variety of the multimer structures. Since the extent of multimer sizes is parallel to the activity of VWF, it is possible to regulate the VWF activity in human plasma using a VWF-specific venom metalloproteinase such as kaouthiagin.

Snake venoms have been reported to stimulate the release of plasminogen activators from endothelial cells. This activity was most pronounced in the venoms of the rattlesnakes *C. atrox* and *C. adamanteus* [68]. A venom plasminogen activator (TSV-PA) (Table 1) [18,19] may act through a different mechanism from that of mammalian plasminogen activators and may be not inhibited by most SERPINs, suggesting that it may have certain advantages over the mammalian enzymes in the clinical use.

More detailed analysis of these unique snake venom proteases including the structural elucidation should provide some useful information for the drug design that could be applied to the medical and pharmacological fields of hemostasis and thrombosis.

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