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Review

Snake venom thrombin-like enzymes: from reptilase to now

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Abstract. The snake venom thrombin-like enzymes (SVTLEs) comprise a number of serine proteases functionally and structurally related to thrombin. Until recently, only nine complete sequences of this subgroup of the serine protease family were known. Over the past 5 years, the primary structure of several SVTLEs has been characterized, and now this family includes several members. Of particular interest is their possible use in pathologies such as thrombosis. The aim of the present review is to summarize the state of the art concerning the evolutionary, structural and biological features of the SVTLEs.

Key words. Snake venom; thrombin; fibrinogen; platelets; primary sequence; serine protease.

Introduction

Snake venoms contain a variety of proteolytic enzymes affecting the host coagulation process [1–3]. One of these enzymes responsible for in vitro blood-clotting activity present in several snake venoms is a serine protease which resembles at least in part thrombin, a multifunctional protease that plays a key role in coagulation. Hence these enzymes are denominated snake venom thrombin-like enzymes (SVTLEs), and are widely distributed in the venoms of several genera [4, 5].

Since the coagulation studies on reptilase in 1957 until the last inventory of these enzymes in 1998, over 40 thrombin-like enzymes had been isolated and characterized [5, 6]. However, only 9 SVTLEs were totally sequenced. In the last 5 years a large number of studies, including molecular cloning, have led to the identification of a large number of SVTLE primary structures that have been accurately characterized.

At present, more than 30 SVTLE primary structures are known that share the active site sequence motif, in which important residues (His57, Asp102, Ser195) are highly conserved. Based on the primary structure analysis of these enzymes and their susceptibility to classical serine protease inhibitors, the evolutionary relationship of SVTLEs with their mammalian counterpart trypsin is clearly indicated.

Current studies show that SVTLEs may affect many parts of the haemostasis ranging from activation of coagulation factors to induction of platelet aggregation [7, 8]. Some SVTLEs have been found to have biological activity not only in the haemostatic process but also in nervous and complement systems [9, 10].

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As in the previous inventory [5] there is still no standard in the biological assay described for SVTLEs, and a large number of inhibitors and coagulation factors have been tested. To standardize these experiments for all SVTLEs is a challenging issue that lies ahead.

In this review, the data available in the literature will be used to give a fair idea about levels of activity and/or susceptibility exhibited by these enzymes. This review particularly concentrates on how the knowledge of new snake venom thrombin-like enzymes can advance our understanding of this serine protease subtype and its potential therapeutical use.

SVTLE distribution

The last inventory [5] showed that SVTLEs were identified mainly in venoms from snakes of the ***Crotalinae*** subfamily, spread over several species from various genera, including Agkistrodon (*A. acutus, A. bilineatus*, *A. caliginosus, A. c. contortrix, A. halys blomhoffii, A. h. pallas* and *A. rhodostoma*), Bothrops (*B. asper, B. atrox, B. barnetti, B. hyoprosus, B. insularis, B. jararaca, B. jararacussu, B. moojeni* and *B. pictus*), **Crotalus** (*C. adamanteus, C. atrox, C. durissus terrificus, C. h. horridus, C. r. ruber, C. v. helleri* and *C. v. oreganos*), **Lachesis** (*L. muta, L. m. muta, L. m. noctivaga, L. m. rhombeata* and *L. m. stenophrys*), **Trimeresurus** (*T. flavoviridis, T. gramineus, T. okinavensi* and *T. s. sumatranus*) and also in other subfamilies; ***Viperinae*** (*Cerastes cerastes*, *C. vipera* and *Bitis gabonica*) and ***Colubrinae*** (*Dipholidus typus*). Since then, several SVTLEs were described in the literature not only in the same species, such as *A. caliginosus* [11], *A. c. contortrix* [12], *A. halys blomhoffii* [13], *A. h. pallas* [14, 15], *B. atrox* [16, 17], *B. jararaca* [18], *B. jararacussu* [19], *T. stejnegeri* [20] and *T. flavoviridis* [21–23], but also in different species, e.g. *A. halys* [24], *A. h. brevicaudus stejneger* [25], *A. h. ussuriensis* [26, 27], *A. saxatilis* [28], *B. alternatus* [29], *B. lanceolatus* [30], *T. mucrosquamatus* [31], *T. elegans* [32, 33], *T. jerdonii* [34, 35], *T. stejnegeri* [36] and different genus ***Gloydius*** (*G. ussuriensis* and *G. shedaoensis*) [37, 38]. As argued by Tsai and co-workers, some controversy still has arisen with regard to the real identity of the major SVTLE present in the venom of *A. acutus* [39–42]. Although with substantial reservations, the present review kept a non-judgmental primary sequence list of some SVTLEs, including those proteins (see [39–42] for additional information).

This distribution is in agreement with the common symptoms of envenomation by these snakes, which include microcirculatory and macrocirculatory thrombosis, unmeasurable prothrombin (PT) and partial thromboplastin times (PTT), and reduction of the level of or even undetectable fibrinogen in the victims [1, 43–46].

Common features of SVTLEs

The snake venom glands are assumed to originate from the submaxillary glands that are rich in glandular serine proteases, mainly kallikrein. In evolution it is supposed that the functions of these enzymes have altered but the overall structure has undergone only minor modifications [47]. The genes for SVTLEs are members of the trypsin/kallikrein gene family, like them comprised of five exons and four introns. The mature enzyme is encoded by exons 2 to 5, while the putative catalytic triad residues are encoded by separate exons [42, 48].

Comparison of the primary sequences of SVTLEs with those of classical serine proteases such as chymotrypsin, thrombin, kallikrein and trypsin identifies SVTLEs as serine proteases according their degree of similarity (table 1). The catalytic domain is the main conserved region on these enzymes, being directly responsible for this similarity. From another point of view, the degree of similarity of the primary structure of SVTLEs with these classical en-

Table 1. Similarity of 30 SVTLEs to classic known serine proteases (chymotrypsin, α-thrombin, kallikrein and β-trypsin), a plasminogen activator from the snake *Trimeresurus stejnegeri* (TSV-PA) and within the SVTLE subfamily.

Enzymes	No. of residues	Similarity[a] (%)			Biological activity
		Min.	Max.	Mean	
Chymotrypsin	245	24	31	28	hydrolysis of dietary protein molecules containing large aromatic residues at the scissile bond
α-Thrombin	259	26	33	31	clotting fibrinogen
Kallikrein	237	34	40	38	release of bradykinin
β-Trypsin	224	31	44	41	hydrolysis of dietary protein molecules containing Arg at the scissile bond
TSV-PA	234	53	74	66	plasminogen activation
SVTLEs	233 ± 10	51	98	68	clotting fibrinogen

[a] Thirty SVTLE primary sequences, showed in figure 1, were aligned with chymotrypsin, thrombin, kallikrein, trypsin and TSV-PA, and the degree of similarity was calculated using CLUSTAL W (http://www.ebi.ac.uk/clustalw).

zymes also reveals several substitutions in their overall structure. SVTLEs share a weak sequence identity with chymotrypsin (24–31%), a pancreatic serine protease, and thrombin (26–33%) yet are functionally analogous to it (table 1 and fig. 1). SVTLEs are more similar to kallikrein (34–40%) and the highly nonspecific trypsin (31–44%), based not only on the catalytic domain but also on other conserved features, e.g. five topologically equivalent disulfide bonds (table 1 and fig. 1). The sequence homology of SVTLEs is greatest with TSV-PA (53–74%), a serine protease from *Trimeresurus stejnegeri* venom that activates plasminogen and covers 51–98% within its own subfamily (table 1 and fig. 1) [49].

SVTLEs contain 12 cysteine residues at conserved positions (fig. 1), except for Dav-KN, a serine protease deduced from complementary DNA (cDNA) sequences obtained from *Deinagkistrodon acutus* glands in which Cys201 is replaced by Ser201 and messenger RNA (mRNA) probably represents a pseudogene (not shown) [42]. The absence of free thiols in SVTLEs led to the assumption that all 12 cysteines are linked in disulfide bonds. These pairings were confirmed experimentally on bilineobin from *Agkistrodon bilineatus* and contortrixobin from *A. contortrix contortrix* [12, 50]. Therefore, SVTLE disulfide bridges are predicted to be the pairs of cysteine residues 22–157, 42–58, 91–250, 136–201, 168–182 and 191–220 and as found in the crystallographic structure of TSV-PA – the only crystal structure of a venom serine protease currently available [51]. Treatment with a disulfide reducing agent such as β-mercaptoethanol or dithiothreitol (DTT) commonly affects SVTLE biological activities such as described for flavoviridiobin from *Trimeresurus flavoviridis* [5, 22]. These data reveal the important role of these disulfide bonds in the stabilization of SVTLE native structure. In fact, the Cys136–Cys201 bridge is indicated as essential

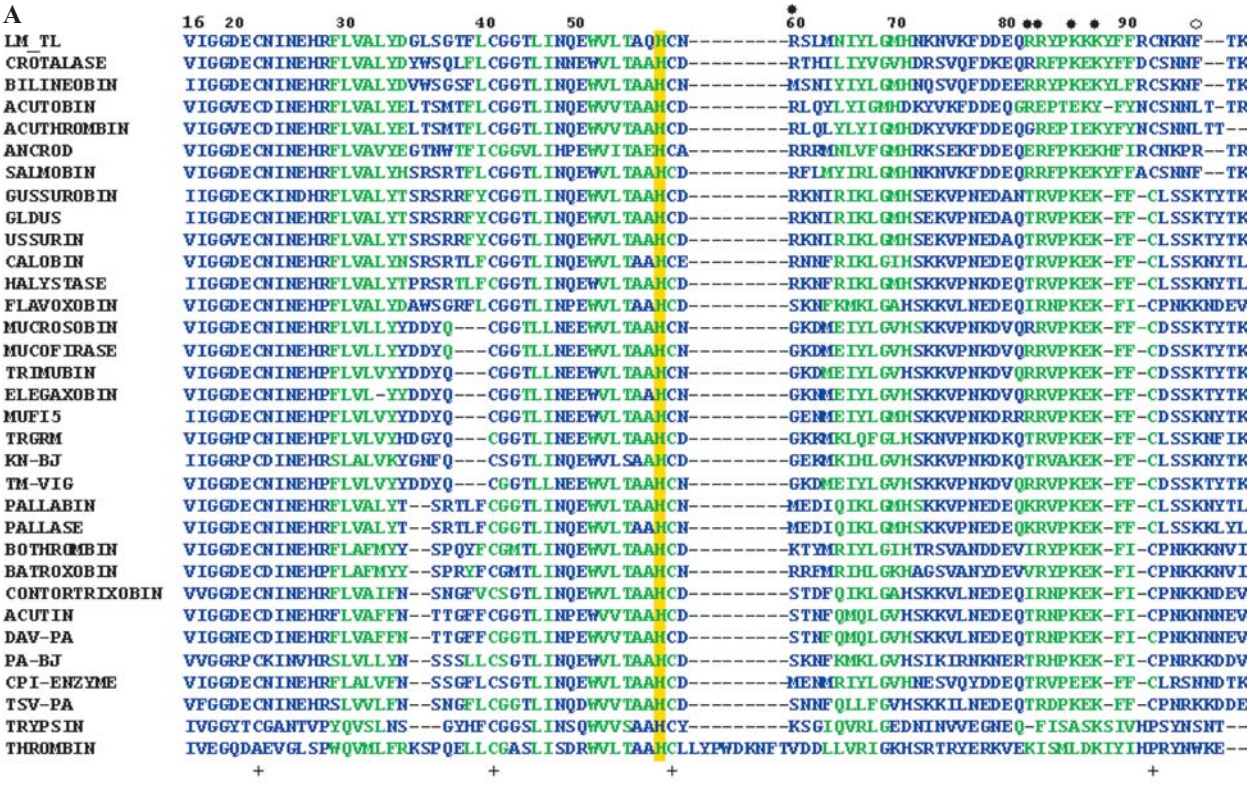

Figure 1 A–C. Multiple sequence alignment of 30 SVTLEs from different snake venoms, TSV-PA, trypsin and thrombin using CLUSTAL W (http://www.ebi.ac.uk/clustalw) [121]. The abbreviations are thrombin-like enzymes from *Lachesis muta* (LM-TL), *Trimeresurus mucrosquamatus* (TM-VIG), *Trimeresurus gramineus* (TRGRM gi:13959620) and *Gloydius ussuriensis* (GLDUS), mucrofibrase 5 from *Trimeresurus mucrosquamatus* (MUFI5 gi:13959635), capillary permeability-increasing enzyme-2 from the venom of *Agkistrodon caliginosus* (CPI-ENZYME gi:13959623), serine protease (PA-BJ) and kinin-releasing and fibrinogen-clotting serine proteinase (KN-BJ) from the venom of *Bothrops jararaca,* and serine protease from *Deinagkistrodon acutus* venom (DAV-PA). Dashes represent gaps to bring the sequences to better alignment. The amino acids of the catalytic triad (H57, D102, S195) are shown highlighted in yellow. On the bottom of the alignment cysteine residues are marked by (+). The asterisk denotes the position of S1 (D189), S2 (G216) and S3 (G226) of the binding pocket, crucial for substrate specificity. The spatially analogous anion binding site and aryl binding site are marked by (●) and (○), respectively, at the top. The secondary structure was predicted by using the Jnet prediction method (http://www.compbio.dundee.ac.uk/~www-jpred/submit.html) [122], and it is indicated in the sequence; α helices are highlighted in red, β strands in green and extended region in blue. Numbering is according to chymotrypsinogen [79].

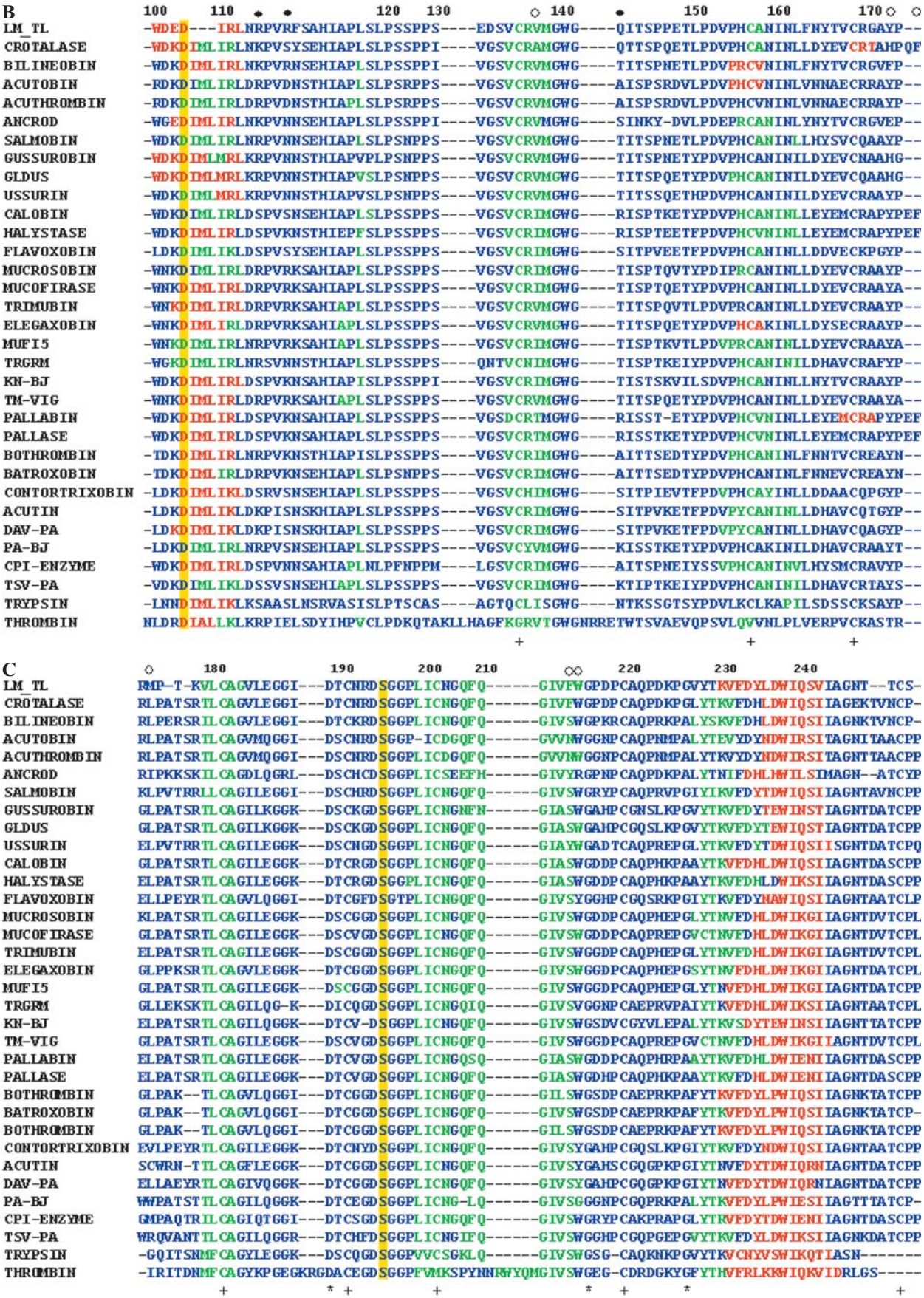

Figure 1 B, C. (continued)

for stabilization of the snake venom serine protease structure in studies of TSV-PA [51], and three-dimensional (3D) models of LM-TL from *Lachesis muta* [52], crotalase from *Crotalus adamanteus* [53] and other SVTLEs [54, 55].

The secondary structure prediction for SVTLE sequence reveals several β-sheet structures, inferring a hypothetical β/β hydrolase fold typical of serine proteases from the chymotrypsin family (figs 1, 2A) [56, 57]. Although the central region surrounding the catalytic triad is structurally highly conserved, the surfaces of SVTLEs may show considerable shape differences given the wide variations in loop sequence and length (fig. 1). These variations should play an important role in SVTLE activity and specificity as described for mammalian serine proteases [58]. SVTLEs such as batroxobin from *Bothrops atrox* venom [59] and plasminogen activators (TSV-PA), similar to their mammalian counterpart, e.g. trypsin, require the exchange of amino acids in multiple portions of the protein in order to convert their specificity [60, 61]. Studies on the substrate specificity of TSV-PA by site-directed mutagenesis revealed that when parts of its sequence were replaced by the equivalent batroxobin residues, none generated either fibrinogen-clotting or direct fibrinogenolytic activity, having little effect on the specificity of TSV-PA for plasminogen [60].

SVTLEs are known as single-chain enzymes such as balterobin, a 30-kDa SVTLE from *Bothrops alternatus* [29], and jerdonobin, a 38-kDa SVTLE from *Trimeresurus jerdonii* [34]. Nevertheless, some of them present two chains, such as RP-34, an enzyme from *Cerastes cerastes* venom (2 × 48.5 kDa) [62].

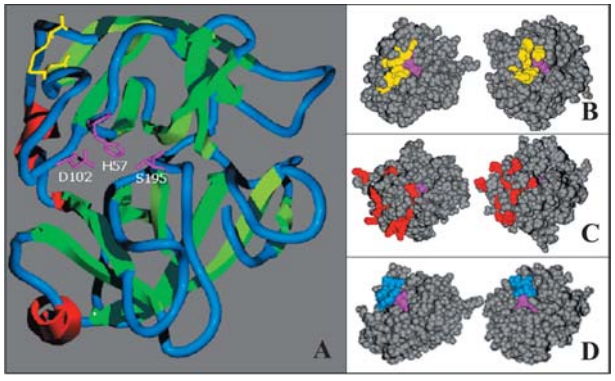

Figure 2. Structural features of SVTLEs represented by the 3D model of LM-TL, a thrombin-like enzyme from *Lachesis muta* venom [52]. (*A*) Secondary structure diagram of LM-TL revealing the β/β hydrolase fold typical of serine proteases from the chymotrypsin family and the spatial position of the catalytic triad (H57, D102, and S195) (pink). The extended region is represented in blue, α helix in red and β strand in green. The LM-TL additional disulfide bridge characteristic of snake serine protease is indicated in yellow. (*B*, *C* and *D*) Space-filling representation of LM-TL (left) and β chain of thrombin (right), comparing their hydrophobic sites (yellow) (*B*), LM-TL positively charged patch and thrombin anion binding exosite I (red) (*C*), and LM-TL 90 and thrombin 60's loops (blue) (*D*). The catalytic triad (H57, D102 and S195) is in pink.

Most of these serine proteases are likely to be glycoproteins, each containing a few Asn-N-linked glycosylation sites in nonhomologous positions to one another in the sequence. The carbohydrate content of SVTLEs also varies widely from 0 to 30% and is characteristic of N-linked glycans. Fucose, hexose, sialic acid, N-acetyl-D-glucosamine (GlcNAc) galactose, mannose and N-acetyl-neuraminic acid (NeuAc) are also found [5, 19, 28, 33, 42]. Although present in many SVTLEs, the specific importance of the carbohydrate moiety on the structure-function relationship is not yet established. In the case of elegaxobin II from *Trimeresurus elegans* venom, N-deglycosylation affects its interaction with macromolecules (fibrinogen and kininogen) but not with small molecules, e.g. *p*-tosyl-L-arginine methylester (TAME) [33]. This result could suggest the direct participation of the carbohydrate region of this enzyme on the recognition of these substrates. Nevertheless, in other serine proteases, glycans are important structures for protein stabilization rather than for the catalytic function of the venom enzymes [63]. Further studies will be necessary with other SVTLEs to define precisely the importance of carbohydrate for their final active structure.

Catalytic and binding sites

All sequenced SVTLEs described so far present several important positions conserved as the catalytic triad residues (His57, Asp102 and Ser195), and the primary (S1) and secondary (S2) specificity sites (Asp189 and Gly216 respectively) (fig. 1). The presence of these specificity subsites guarantees the interaction of the SVTLEs with basic P1 residues of substrates such as TAME and α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) [5, 12, 31, 42]. SVTLE primary structure lacks amino acid residue insertions around the active site cleft (S1 loop) in comparison with thrombin (segment 187–190) (fig. 1) [64–66]. Therefore this feature, which allows the large substrate side chain access to the base of the thrombin catalytic site, is not present on SVTLEs. Furthermore, the S1 loop sequence of SVTLEs conserves the hydrogen bond acceptor/donor group (a hydroxyl group of Ser or Thr residues) at the 190 position similar to trypsin (Ser190) and in contrast to thrombin (Ala190) (fig. 1).

The tertiary specificity site (S3) is occupied by Gly226 on most SVTLEs, being replaced by a small residue, alanine, on some of the SVTLEs analyzed (fig. 1). This S3 mutation should not interfere with the conservation of the overall tertiary fold of the catalytic site (see fig. 2A) but could be involved in the SVTLEs' higher specificity for Pro residues than for Gly and Phe at the P2 position of substrates [20, 42, 67]. Residues 192 and 193 have also

been recognized as key residues affecting snake serine protease specificity for the P2 subsite [68–70]. In fact three regions (residues 82–99, 192–193 and 215–217) have been shown to bear unique substitutions that might be subtype specific [12, 42]. These regions happen to be the known substrate binding subsites of snake venom serine protease, as shown by crystallographic studies [51]. In the course of divergence, these interesting mutations are observed in some SVTLE flanking sequences, such as the basic residue arginine at the 193 position instead of glycine on thrombin and trypsin or phenylalanine on TSV-PA (e.g. LM-TL, crotalase, salmobin from *Agkistrodon halys*, acutobin from *Deinagkistrodon acutus*, acuthrombin from *A. acutus* and bilineobin from *A. bilineatus*) (fig. 1) [24, 40, 42, 50]. Specifically, residues in the 215 and 217 positions are entirely important since they have emerged as key residues in controlling the interaction of thrombin with fibrinogen. The mutation of these residues in thrombin compromises the procoagulant function of the enzyme up to 500-fold [71]. Position 215 is strictly conserved in SVTLE sequences, but 217 diverges widely in most of the SVTLEs aligned in figure 1. Consequently, these substitutions may compromise the specificity and efficiency of the catalytic mechanism of this serine protease subfamily in the same way as it does thrombin.

A number of SVTLEs show several substrate specificities, such as acting on many different molecules, e.g. fibrinogen and the factors of coagulation cascade. Their folding and structural features should imply these alterations. Thrombin is able to interact with fibrinogen and other important molecules using two different regions called anion binding exosites (ABE-I and ABE-II) as well as a hydrophobic site also called the aryl binding site. The latter is conserved on thrombin of several species, apparently pointing to the importance of this site for SVTLE activity [72]. Crotalase and LM-TL models describe a fully solvent exposed, hydrophobic region of a surface loop mapping near one edge of the active site of these enzymes that seems to be involved in their interaction with substrate (fig. 2B). According to LM-TL and crotalase models, the residues that can comprise this region are Phe95, Trp99, Tyr172, Phe174, Leu176, Phe214 and Trp215, which are spatially analogous to Tyr60A, Trp60D, segment 97–99, Ile174 and Trp215 of thrombin (fig. 2B) [52, 53, 73]. This region seems to be represented in some SVTLEs, though not all positions are conserved in this subfamily such as Phe95, 174 and 214 [74] (fig. 1).

ABE-I is a key site for thrombin molecular recognition and subsequent catalysis of its natural substrates [64–66]. It binds to fibrinogen, platelet receptors (PAR-1 and PAR-3, platelet GPIb), the C-terminal end of hirudin, a leech-derived inhibitor, and thrombomodulin. Thrombin ABE-II binds to certain glycoaminoglycans such as heparin. None of these exosites is conserved in SVTLEs. Only an alternative cationic region homologous to ABE-I is observed in SVTLEs that can comprise arginines 60, 81, 82, 107, 110 and 113, and lysines 73, 76, 85, and 87 indicated by the LM-TL and crotalase models, instead of the thrombin arginines 31, 67, 73, 75, 77 and 110, and lysines 32, 70, 81 and 109 (figs 1, 2C). The multiple structural alignment points to a group that seems to conserve most of these residues formed by ussurin from *Agkistrodon halys ussuriensis* [27], gussurobin from *Gloydius ussuriensis* [37], ancrod from *A. rhodostoma* [75], LM-TL, crotalase, calobin [76], salmobin [24] and acutobin [42] (fig. 1). The ineffectiveness of the thrombin inhibitors upon these molecules suggests that this positively charged patch is not capable of interacting with these ABE-dependent inhibitors and/or it is not involved on fibrinogen recognition. There is no doubt that the structural landscape around the primary specificity pocket of both SVTLEs and thrombin are different, and therefore their molecular mechanisms for biorecognition are expected to be diverse. The theory is that fibrinogen recognition mechanism by these SVTLEs could use both aryl and positively charged sites, but it may occur in a different complex manner.

Experimental and theoretical data have shown that SVTLEs are not allosterically regulated by sodium ions such as trypsin [12, 52]. This result was expected due to the conservative presence of a proline at position 225 in that serine protease subfamily, instead of tyrosine presented in thrombin (fig. 1) [77]. Analyses of the catalytic activity of a number of serine proteases with Tyr225 or Phe225 were found to discriminate among monovalent cations and showed maximal catalytic activity in the presence of sodium ions. On the other hand, proteases with Pro225, such as trypsin or SVTLEs, show loss of discrimination among monovalent cations and, accordingly, no allosteric regulation [78].

Interactions with classical serine protease and thrombin inhibitors

Common serine protease inhibitors such as diisopropyl fluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF) inhibit SVTLEs, confirming the serine protease function of these enzymes [5]. Similar to thrombin, these enzymes are usually insensitive to classical trypsin inhibitors such as BPTI, a Kunitz-type trypsin inhibitor with a single binding site (P1 = Lys15), and α1-antitrypsin [5]. Despite the fact that SVTLEs lack the typical Tyr-Pro-Pro-Trp characteristic insertion in the 60 and 148 loops of thrombin [64–66, 79], other loops may appear having the same restriction role (fig. 1). Docking studies using the LM-TL model predicted unfeasible interactions of its extended 90's loop (Phe95 and Trp99) as well as other residues (Lys93, Arg193 and Ser250) with BPTI

[52]. According to the analysis of the SVTLE primary sequence, the 90's loop is a conserved region, suggesting that it could be a restriction loop presented in this serine protease subfamily (figs 1, 2D). It is interesting to note that this loop would also partially participate in the hypothetical SVTLE aryl binding site (figs 1, 2A and 2D).

As expected, common thrombin inhibitors such as antithrombin III, hirudin and heparin do not inhibit most thrombin-like enzymes [5]. The lack of ABE-I and II in SVTLEs apparently confirms these results, since these inhibitors are recognized by thrombin using these exosites. The small group that is affected by these inhibitors usually has no determined primary structure similar to thrombocytin, a SVTLE whose biological activity is affected by heparin with antithrombin III [43]. A known exception is ancrod, a SVTLE from *A. rhodostoma* inhibited by antithrombin III, whose primary sequence is already identified. Although it is expected that the ancrod sequence presents some direct structural feature to clarify its mechanism of interaction with this inhibitor, the sequence provides no clue as to how this mechanism works.

Biological properties of SVTLE

Similar to thrombin, SVTLEs can catalyze the last in the series of narrowly specific proteolytic reactions that lead to clotting of the blood protein fibrinogen [5, 55, 80, 81]. The unique exception is PA-BJ, a platelet-aggregating enzyme from *Bothrops jararaca* venom that catalyzes the hydrolysis of several *p*-nitroanilide peptide substrates containing Arg or Lys at the scissile bond, but not fibrinogen [82].

Despite the conserved active site sequence motif, there are some differences in SVTLE catalytic activity upon fibrinogen as compared with thrombin. Some SVTLEs are able to cleave both Aα and Bβ chains of fibrinogen, releasing fibrinopeptide A (FPA) and B (FPB), but most of them preferentially act upon only one of the fibrinogen chains (Aα or Bβ) [5]. Based on this feature, SVTLEs have been classified as FP-A, FP-B or FP-AB [1], or venobim A, B or AB [83]. In this review we suggest that this serine protease subfamily use the SVTLE designation, since venobim does not directly indicate the class of these enzymes, compared with other families such as the plasminogen activators (PAs), and FP designates the reaction product not the enzyme (fig. 3).

Therefore, SVTLEs could be grouped into three categories; (i) SVTLE-AB, composed of enzymes that, like thrombin, trigger the clotting of fibrinogen by hydrolytic release of both fibrinopeptide A and B, e.g. jararacussin from *B. jararacussu* venom [19], THLE from *Trimeresurus flavoviridis* [23] and Kangshuanmei from *Agkistrodon halys brevicaudus stejneger*i [25]; (ii) SVTLE-A, characterized by cleaving only the Aα chain, such as ancrod [84] and flavoxobin from *T. flavoviridis* [85]; and (iii) SVTLEs-B that act only upon the Bβ chain, e.g. Contortrixobin from *Agkistrodon contortrix contortrix* [12] and okinaxobin I from *Trimeresurus okinavensis* venom [87] (fig. 3).

The species specificity of SVTLE clotting activity is not well characterized, with the exception of habutobin from *T. flavoviridis* and elegaxobin from *T. elegans* [88, 89]. Both enzymes act only upon rabbit fibrinogen, whereas they show no effect on human and bovine fibrinogens. In the rabbit fibrinogen-fibrin conversion induced by them, only fibrinopeptide A is preferentially released, revealing their SVTLE-A profile. Habutobin also does not recognize monkey, dog, rat, or guinea pig fibrinogens [88]. It is interesting to note, and perhaps not surprising, which SVTLE did not clot its own nor any other snake plasma in contrast to human thrombin. These data confirm that SVTLEs are not directly equivalent to either human or snake thrombin [90–92].

Based on the literature available, excluding some few exceptions, most snake venom thrombin-like enzymes studied do not activate factors from the coagulation cascade or only act upon factor XIII (FXIII) [5]. Factor XIII, an important zymogen activated by thrombin [64–66], is a fibrin cross-linker that makes the clot resistant to rapid removal by the blood circulation (fig. 4). This coagulation cascade factor does not recognize the abnormal fibrin polymers formed by SVTLEs [93, 94]. Consequently, these aberrant fibrin monomers are easily dispersible and more susceptible to plasmin proteolysis. Some SVTLEs are able to activate this factor, including contortrixobin, acuthrombin and cerastocytin from *Cerastes cerastes,* as well as other serine proteases purified from the genera **Agkistrodon**, **Bitis**, **Bothrops**, **Cerastes** and **Trimeresurus** (fig. 4) [5]. Among the interesting exceptions that activate other factors, we can cite contortrixobin, a 25.5-kDa protease from *Agkistrodon contortrix contortrix.* [12]. This SVTLE is able to activate factor V, a glycoprotein that plays an important role in the procoagulant and anticoagulant pathways [95], with a rate 250–500-fold lower than thrombin (fig. 4). This sin-

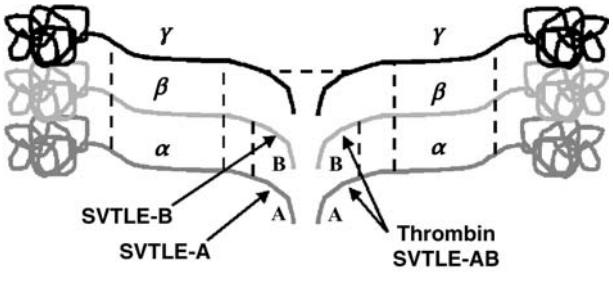

Figure 3. Comparison of the catalytic pattern of thrombin and SVTLEs upon fibrinogen. The symbols α, β and γ represent the chains of fibrinogen, while letters A and B refer to the fibrinopeptides that are released by their hydrolytic action.

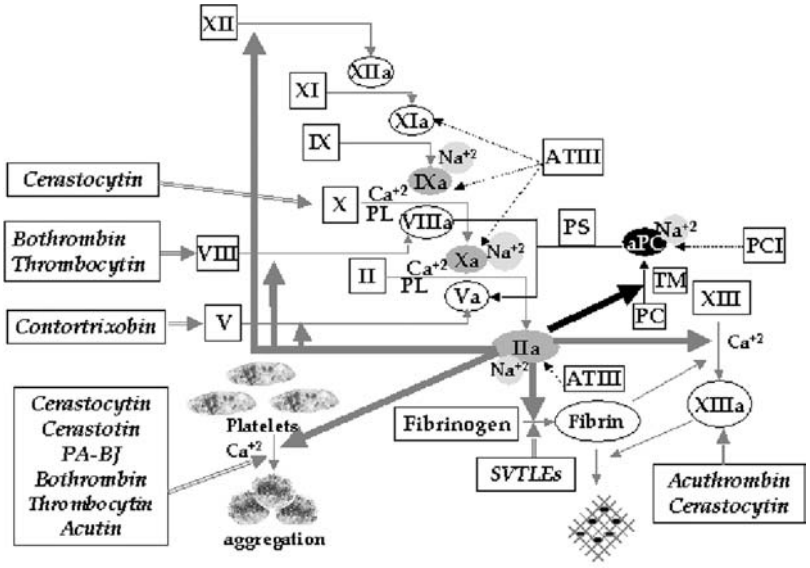

Figure 4. Schematic representation of the effect of the SVTLEs upon the intrinsic pathway of the blood coagulation cascade and platelet aggregation. Major interactions and regulatory feedbacks that lead to the conversion of zymogens (rectangles) to active proteases (ovals) are shown. SVTLEs are represented by blocks, procoagulant reactions by gray, reactions with protease inhibitors by black dotted lines, and the protein C-mediated anticoagulant pathway by a black line. The vitamin K-dependent proteases thrombin (factor IIa), factors Xa, IXa, VIIa and activated protein C all possess a functional Na binding site (light gray) that is required for efficient catalysis. Abbreviations: aPC, activated protein C; ATIII, antithrombin III; PC, protein C; PCI, protein C inhibitor; PL, phospholipids; PS, protein S; TM, thrombomodulin. This schematic representation was adapted with permission from Di Cera et al. [64].

gular behavior is also observed for bothrombin and thrombocytin from *B. jararaca* and *B. atrox,* respectively, which activate factor VIII [43, 96]. In the case of bothrombin, its efficiency is also low, about 950 times less than that of α-thrombin (fig. 4). Cerastocytin is also able to activate factor X, which is an interesting and rare feature in the SVTLE subfamily [97].

Thrombin-like enzymes from snake venoms are potentially interesting tools for investigating the complex mechanism of platelet aggregation. Some SVTLEs (though not all) induce platelet aggregation in the presence of exogenous fibrinogen, often with much lower potency than thrombin [2, 5, 7, 98]. As a consequence, the platelet-activating potency of snake venom thrombin-like enzymes has been correlated with their effectiveness on the retractility and elasticity of the clots. Cerastotin from *Cerastes cerastes* venom aggregates platelets in the presence of exogenous fibrinogen. Its activity is not inhibited by a monoclonal antibody anti-GPIIb-IIIa that blocks fibrinogen binding, but by a monoclonal antibody directed against glycoprotein Ib, which also specifically inhibits induced agglutination by ristocetin (fig. 4) [99]. On the other hand, some SVTLEs do not need fibrinogen to aggregate platelets. PA-BJ from the venom of *Bothrops jararaca* is a platelet aggregation inducer that causes a release reaction without clotting fibrinogen. Acutin from *Agkistrodon acutus* venom, and batroxobin and thrombocytin from *Bothrops atrox* venom also induce aggregation and ATP release of washed rabbit platelets [7]. The aggregating activity of PA-BJ, thrombocytin, acutin and batroxobin is 10, 10^2, 10^4 and 10^5 times less potent than that of thrombin, respectively [7, 8]. The major cleavage site on PAR1 by thrombin, PA-BJ and thrombocytin is Arg41-Ser42, and both PAR1 and PAR4 seem to mediate the effect of these enzymes on platelets (fig. 4). Platelet aggregation induced by these SVTLEs could be inhibited by heparin with antithrombin III, while that by acutin or batroxobin could not [7]. Recent analysis of the effect of two snake venom thrombin-like enzymes upon human thrombin receptors revealed that okinaxobin I isolated from *Trimeresurus okinavensis* is able to stimulate phosphoinositide turnover and DNA synthesis [100]. Okinaxobin I ability is 4000 times less potent than that of α-thrombin, thus suggesting that the interaction of snake venom thrombin-like enzymes with human thrombin receptors is less efficient than thrombin.

SVTLEs may also present other biological activities totally distinct from the most familiar of the many actions of thrombin. An interesting example is LM-TL, a SVTLE-A from *Lachesis muta* venom composed of a single polypeptide chain, which when injected into the tail veins of mice induces temporary episodes of opisthotonos and rapid rolling around the long axis of the animals [101, 102]. Curiously enough, a homologous mammal serine protease called neuropsin expressed mainly in the nervous system is suggested to be involved in kindling epileptogenesis and hippocampal plasticity. Neuropsin

exhibits chimeric features between trypsin and nerve growth factor-gamma, a member of the kallikrein family. Similar to LM-TL, this protease forms disulfide bonds corresponding to those of trypsin, and its active site is also restricted to smaller residues or proline at the P2 position of substrates [52, 103]. The superposition of the LM-TL model with neuropsin crystal structure shows a root mean square (RMS) of about 0.73 Å, and the overall folds of their β-β-barrel structures are superposable (not shown). These similarities raise questions about their mechanism in the mammalian nervous system, which could help to understand their structural relationship and the design of specific inhibitors. Studies involving the effect of SVTLEs on the nervous system show the neuroprotective mechanism of batroxobin on spatial memory disorders of left temporal ischemic rats, and its effects on the expression of c-Jun, neural cell adhesion molecule (NCAM), HSP32 and HSP70 [9, 104, 105].

Included in these activities, several SVTLEs have kinin-releasing activity, leading to a hypotensive effect [2, 63, 106]. Generally, the kinin that is released from plasma kininogens by most snake venom enzymes, such as halystase from *A. halys blomhoffii* [17], flavovilase from *Trimeresurus flavoviridis* [21] and KN-BJ from *B. jararaca* venom [107], is bradykinin. Curiously, elegaxobin II from *Trimeresurus elegans* venom is the unique SVTLE cited so far that is able to release Lys-bradykinin from plasma kininogens [33]. This serine protease subfamily can also be found activating the human alternative complement system. Flavoxobin from *Trimeresurus flavoviridis* acts as heterologous C3 convertase that independently cleaves human C3 and kick-starts the complement cascade [10].

Taken together, these totally diverse biological results with SVTLEs show that many fruitful avenues of investigation are now open. Much needs to be understood about the substrate specificity of these SVTLEs in diverse tissues and how their enzymatic action mediates biological effects.

Evolutionary aspects

Evolution is about homology, that is the similarity due to common ancestry [56, 57, 108]. The evolution of snake venom proteinases is not fully studied, and evidence to determine SVTLE ancestry is lacking. Molecular phylogenetic trees have been used to represent the evolutionary past and the probability of a common ancestry to SVTLEs and other classical serine proteases [12, 42, 109, 110]. All the data obtained so far suggest that the amino acid sequences of venom gland serine proteases have diversified in an accelerating manner [47]. According to them, like PLA2, the original form of SVTLEs should have a specific activity. Based on the complete amino acid sequence of these enzymes, the literature describes the independent and parallel evolution of three major enzyme subtypes, the coagulating enzymes (CLs), the PAs and the kininogenases (KNs) [12, 42, 47]. In order to discuss the evolutionary relationship among trypsin, thrombin, TSV-PA and the SVTLEs, we constructed a phylogenetic tree with the sequences of 30 SVTLEs, also including TSV-PA and these two classical serine proteases (fig. 5).

Our tree topology results are congruent with previous comments in the literature that point to a common ancestor for all these proteases [47, 56, 57, 108]. Similar to previous data described by other authors, the group that presents some analogous thrombin structural features such as the positively charged patch, e.g. ancrod, crotalase, LM-TL and acutobin, are linked together in the subtype closest to thrombin, characterized by being SVTLE-A and highly glycosylated [12, 42]. Meanwhile, elegaxobin was the closest to trypsin in accord with its SVTLE highest degree of similarity (44%). Far from establishing the exact evolutionary biography of this subfamily, this result points to the fact that the more we understand the SVTLE subfamilies, the more we will be able to explore the serine protease family. Recent work on serine proteases described a classification according to evolutionary markers that categorizes serine proteases of the chymotrypsin-like, subtilisin-like and alpha/beta-hydrolase fold clans according to phylogenetic lineages, indicating the relative ages and order of appearance of those lineages [86]. Using the analysis of amino acid and codon usage by highly conserved residues on SVTLE sequence, it is possible to observe that these proteases have kept these primordial evolutionary markers(Ser195:TCN/Ser214:TCN/Pro225). These data suggest that the SVTLE subclass belongs to the most primordial lineage, which includes the proteases present in the middle or downstream cascade of the *Drosophila* dorsal-ventral polarity and the clotting of the horseshoe crab hemolymph.

Therapeutic use

Because of their physiological properties, SVTLEs act in vitro as procoagulants converting fibrinogen to fibrin, while in vivo they cause benign defibrination [1, 45, 111–113]. Since the 1970s, SVTLEs have been extensively studied both by basic researchers and clinicians because of their potential therapeutic use in myocardial infarction, ischaemic stroke and thrombotic diseases [114–116]. Some SVTLEs have being used in the prevention of thrombus formation, also on the foreign surfaces of prosthetic and extracorporeal devices, and in improving the blood circulation in various vascular disorders by reducing blood viscosity. The clinical studies of ancrod from *A. rhodostoma* venom in patients with is-

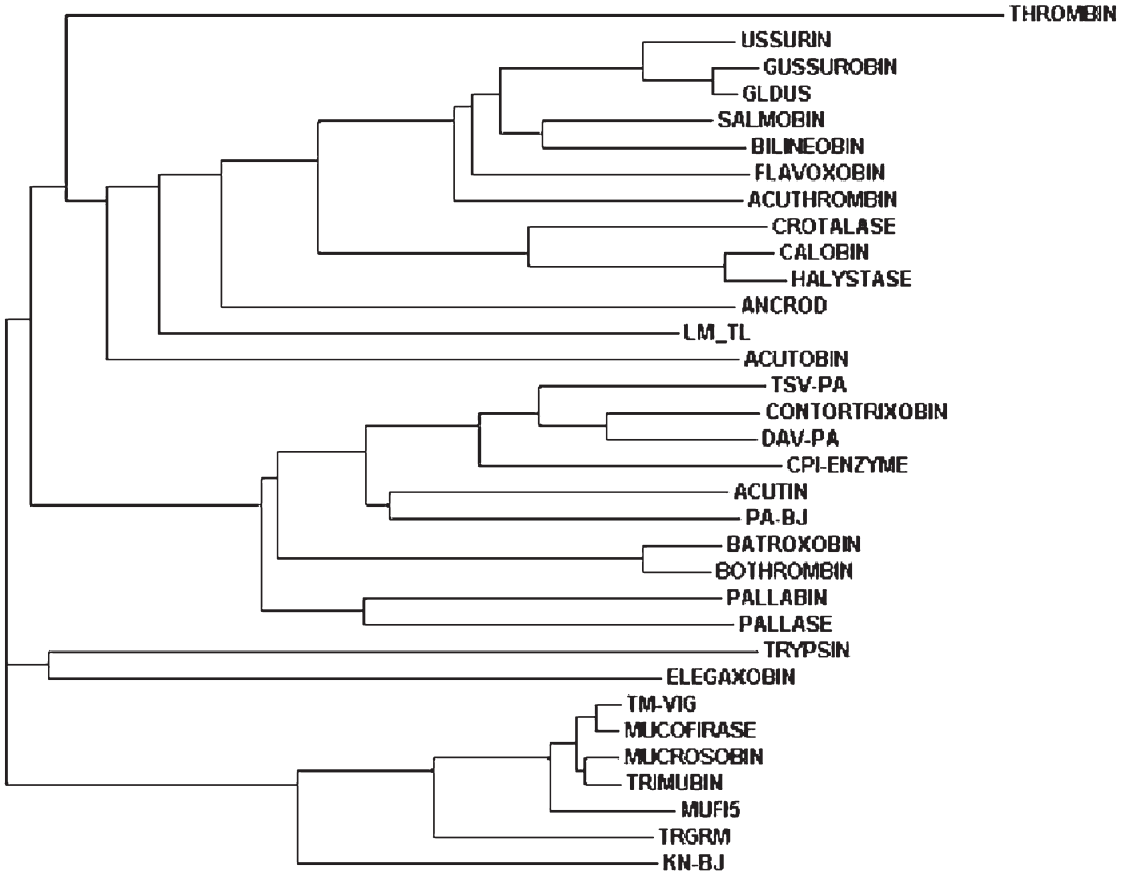

Figure 5. Phylogenetic tree of 30 SVTLE, trypsin, thrombin and TSV-PA sequences using the computer program PHYLIP in the CLUSTAL W package (http://www.ebi.ac.uk/clustalw) [121]. The tree is drawn as a phylogram and should not be looked upon as an attempt to specify the precise sequence or dates of emergence of particular groups of proteases. The tree suggests that the protease domains of the enzymes listed in the crotalase group are most closely related to the protease domain of thrombin. The abbreviations are thrombin-like enzymes from *Lachesis muta* (LM-TL), *Trimeresurus mucrosquamatus* (TM-VIG), *Trimeresurus gramineus* (TRGRM) and *Gloydius ussuriensis* (GLDUS), mucrofibrase 5 from *Trimeresurus mucrosquamatus* (MUFI5), capillary permeability-increasing enzyme-2 from the venom of *Agkistrodon caliginosus* (CPI-ENZYME), serine protease (PA-BJ) and kinin-releasing and fibrinogen-clotting serine proteinase (KN-BJ) from the venom of *Bothrops jararaca,* and serine protease from *Deinagkistrodon acutus* venom (DAV-PA).

chaemic stroke, a pathology that occurs mostly due to embolic or thrombotic occlusion of an artery to the brain, have shown a benefit with ancrod treatment in neurological outcome with only a modest increase in bleeding risk [117]. Batroxobin from the *Bothrops atrox* venom has a good therapeutic effect in ischaemic reperfused rats and clinical practices in vivo. Therefore batroxobin and ancrod are being commercially produced as clinical therapeutical drugs. Three other thrombin-like enzyme preparations have also been commercially available; they are reptilase, crotalase and a thrombin-like enzyme from *Agkistrodon contortrix* [4]. However, the popularized clinical use of thrombin-like enzymes has been limited by immunologic reactions in patients, limited availability of the snake venom and high cost [118].

SVTLEs are also used for fibrinogen and fibrinogen breakdown product assays as well as detecting dysfibrinogenaemias and assaying antithrombin III in samples containing heparin [112, 113]. The SVTLE family has contributed Reptilase, a good reagent used in quantitative determination of fibrinogen, especially in the plasma of patients under heparin treatment. It is possible that other SVTLE members may have applicability as defibrinogenating agents in other chronic and acute diseases. It will be important to identify all the physiological substrates of these enzymes, which would lead to novel clinical applications other than those presently known [119].

Directions for future research

Snake venom thrombin-like enzymes pose several interesting questions at both the evolutionary and structural levels. At the evolutionary level, it is of great interest to understand the genetic events that led to the emergence of molecules with such different biological activities from a

unique ancestor. Equally at the structural level, it is of interest to establish whether SVTLE action on receptors involves similar thrombin domains. Thus, it appears important to establish detailed high-resolution structural information of these enzymes as well as comparative studies using a mutagenesis approach, which could provide insights not only into their biological activities but could also help to understand their very specific recognition of their particular substrates. We are close to knowing these structures since some SVTLEs, such as acuthrombin B [41], jararacussin [19], and bothrombin [120], have recently been crystallized. However, their structural results still remain to be analyzed and made available at the Protein Data Bank. Therefore, there is plenty of information still to be revealed to allow exploitation of the structure-activity relationships and the diversity of these venom proteases, consequently also for improving their therapeutic use. We hope that this update will facilitate new developments in this field and lead to practical applications of these enzymes in treating human diseases.

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