CHAPTER II

LITERATURE REVIEW

1. Serine beta-fibrinogenase in snake venom

Several recent reviews have appeared on snake venom fibrinogenolytic enzyme (Markland, 1991; Meier and Stocker, 1991; Ouyang *et al.*, 1992; Siigur and Siigur, 1992; Hutton and Warrell, 1993; Marsh, 1994). In a recent inventory, Markland (1998) described 67 purified venom fibrinogenolytic enzymes. All of these enzymes are direct acting and do not rely on enzymatic component in the blood for activity. The majority of the fibrinogenolytic enzymes are purified from snakes of the Asian (22/67), North American (23/67), and Central and South American (10/67) *Crotalid* family.

Fibrinogenolytic enzymes have fibrinogen for substrate of enzymes; fibrinogen appears as a large trinodular protein by electron microscopy. The protein contains two symmetric half-molecules which are disulfide-linked. Each half contains three chains designated as Aα, Bβ and γ with molecular weights of 63 500, 56 000 and 47 000, respectively. The fibrinogen molecule has a molecular weight of 340 kDA. (Hettasch and Greenberg, 1994). Fibrinogen contains long stretches of amino acid which are exposed to proteolytic enzymes including the snake venom proteinase. Fibrin, however, has a cross-linked structure and is much less susceptible to proteolysis (Doolittle, 1984).

As indicated previously (Markland, 1991), venom fibrinogenase enzyme may be classified as being either alpha (α)- or beta (β)- chain fibrinogenase. Thus far there have been virtually no reports of fibrinogenolytic snake venom enzyme with cleavage specificity directed solely to the γ- chain of frinogen. Specificity for the alpha (α)- or beta (β)- chain is not absolute since there is substantial degradation of

alternate chain with increasing time. The venom enzymes differ substantially from plasmin which is a serine proteinase and is readily inactivated by plasma serine proteinase inhibitors (SERPINS). Further, plasmin cleaves bonds at the carboxy-terminal side of lysine residues in the α-, β- and γ- chains of fibrinogen, site different than those cleaved by the venom fibrinogenolytic enzyme.

In addition, snake venom appears to contain two groups of fibrinogenolytic enzymes. First, the majority of the fibrinogenolytic enzymes are metalloproteinase with specificity directed preferentially towards the Aα- chain and with some what lower activity towards the Bβ- chain. However, generalizations about chain specificity are not always applicable since there are at least three report of fibrinogenolytic metalloproteinase whose preference is directed to the Bβ- chain. Most of the metalloproteinase are fibrinolytic. The second group of fibrinogenolytic enzymes, the serine proteinase with fibrinogenolytic activity preferentially cleaves the Bβ- chain with lower activity towards the Aα- chain, although there are a number of exceptions to this generalization. Many of them serine proteinase are both fibrinogenolytic and fibrinolytic. However, a number of them are not fibrinolytic. They possess arginine esterase activity and are inhibited by diisopropylfluorophosphate or phenylmethanesulfonyl fluoride, but not by EDTA. The Bβ- chain degrading enzymes have molecular weights in the range of 23 000 to 33 500. Although they are serine proteinases, these enzymes do not appear to resemble thrombin-like (fibrinogen-clotting) serine proteinases from snake venoms which cleave fibrinopeptide A and/or B from fibrinogen.

Serine beta-fibrinogenase or Bβ- fibrinogenase can classify in second group of fibrinogenolytic enzymes, posseses not only caseinolytic activity but also Tos-Arg-OMe esterase activity and its activityis not inhibit by EDTA (Teng *et al.*, 1985). In previous reports, a number of serine beta-fibrinogenase has been studied in several snake venom of *Viperidae* and *Crotalidae*, such as (Hung and Chiou, 2001)

venom of *Crotalus Atrox* (Western Diamonback rattlesnake) has β –Fibrinoginase, Protease II and Protease III. β –Fibrinoginase, P-1, ME-1, ME-2, ME-3 and ME-4 from *Trimeresurus mucrosquamatus* venom (Taiwan habu). β– Fibrinoginase from *Trimeresurus gramineuse* .β– Fibrinoginase from Agkistrodon piscivorus piscivorus venom (eastern cottonmouth). Shedaoenase from *Agkistrodon shedaoenthesis* Zhao venom (Jiao *et al.*, 2005). Fibrinogenase and β– Fibrinoginase from *Macrovipera lebetina* venom (Levantine viper). Moreover, fibrinogenolytic activity found the strongest activity in venoms from *Agkistrodon piscivorus* venom (cottonmouth), *Agkistrodon contortrix* venom (copperhead), Halytases (Matsui *et al.*, 1998) from *Agkistrodon halys blomhoffii*, and brevinase (Lee *et al.*, 1999) from *Agkistrodon blomhoffii brevicaudus* (Markland, 1991; Sapru *et al.*, 1983).

2. Characteristics and Relationships of Serine beta-fibrinogenase

Serine beta-fibrinogenase has been studied by purified venoms in a number of different snakes that were shown different properties in **Table 2**. The venoms were shown to be directly fibrinolytic since they did not require plasminogen for fibrin plate lysis. Fibrinolytic activity was not inhibited by epsilon-aminocaproic acid, soybean trypsin inhibitor, or trasylol. Interestingly, however, serum from humans and other species inactivated the fibrinolytic activity of the snake venoms (Markland, 1991; Ouyang and Teng, 1976).

Moreover, Ouyang *et al.* were studied in *Trimeresurus mucrosquamatus* venom (Taiwan habu). Beta-fibrinogenase of Taiwan habu has also possesses proteolytic activity toward casein and fibrinogen and strong esterase activity toward tosyl-L-arginine but weak anticoagulants as measured by recalcification ime and plasma prothrombin time. β- Fibrinogenase (Taiwan habu) was inhibited by the sera in the following order: rabbit > guinea-pig > dog > human. The different inhibitory potencies of the sera explain the differences of the fibrinogenolytic activities

of enzyme on plasma fibrinogen of various species (Teng, *et al.*, 1985; Ouyang, *et al.*, 1979; Ouyang, *et al.*, 1977). However, Zahra *et al.* were studied of *Crotalus atrox* venom. Beta-fibrinogenase in its lack of activity towards casein, fibrin and an arginine ester (Sapru, *et al*,.1983).

In *Agistrodon* genus were showed Brevinase; was purified from *Agistrodon blomhoffii brevicaudus*, Halystase; from *Agistrodon halys blomhoffii* and Shedaoenase; from *Agkistrodon shedaoenthesis* Zhao. Brevinase consists of two polypeptide chains of 16.5 and 17 kDA linked by disulfide bridge(s). Which were cleaved both the Aα and B β - chains of fibrinogen but did not affect the γ- chains of fibrinogen. It showed β - fibrinogenase activity devoid of fibrinogen clotting and caseinolytic activity. brevinase is a direct-acting fibrino(geno)lytic enzyme different from thrombin-like enzymes (defibrinogenase). Brevinase showed a fibrinolytic activity without degradation of casein, it might have advantage as potential therapeutic agents. Brevinase did not showed any detectable amidolytic activity on the tested chromogenic substrates for trypsin, thrombin, plasmin, and kallikrein. These results suggest that brevinase is a serine protease which has a different substrate specificity from trypsin, thrombin, plasmin, and kallikrein. (Lee, *et al.*, 1999). The other one, Halystase contained the tentative catalytic triad of His43, Asp88 and Ser184 common to all serine proteases and Asp178 in the primary substrate-binding site. Although halystase contained an RGD sequence at residues 181-183, it did not inhibit platelet aggregation induced by ADP or collagen. It hydrolyzed most efficiently a tissuekallikrein substrate, prolylphenylalanylarginyl-4-methyl-coumaryl-7-amide, and released bradykinin from bovine kininogen. Halystase did not coagulate human plasma, but it cleaved the fibrinogen B β -chain at the carboxyl side of Arg42 and cleaved slowly the fibrogen A A chain. Fibrinogen thus treated gradually became insensitive to thrombin. The proteolytic activity was inhibited with diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride or leupeptin. These results indicate that halystase is a serine protease structurally similar to coagulating thrombin-like snake venom proteases, but it specifically cleaves fibrinogen at sites different from thrombin without inducing fibrin clotting, and hydrolyzes kininogen to produce bradykinin, resulting in the reduction of blood pressure (Matsui, *et al.*, 1998).

In *Agkistrodon shedaoenthesis* Zhao was studied by Mang JIAO *et al.* Shedaoenase preferentially cleaved the Aα-chain of human fibrinogen and slowly digested the Bβ-chain. It also showed arginyl esterase activity using Nα-benzoyl-*L*-arginine ethyl ester as a substrate, and some synthetic chromogentic substrates, such as Chromozym PL, S-2266, and S-2160, could also be hydrolyzed. The enzyme activity of shedaoenase could be completely inhibited by phenylmethylsulphonylfluoride and could be little inhibited by the chelating reagent EDTA (Jiao, *et al.*, 2005).

Furthermore, In *Vipera* snake were show Fibrinogenase; were studied a fibrinogenase from *Vipera lebetina* (desert adder) venom by Gasmi *et al.* and Siigur *et al.* were studied beta-fibrinogenase from the venom of *Vipera lebetina* [*Macrovipera lebetina* venom (Levantine viper)]. Fibrinogenase was revealed that it hydrolyzes readily the B beta chain of fibrinogen and the A alpha chain as well as fibrin and casein. it is activated by Ca2+ and Mg2+. Purified fibrinogenase up to a dose of 100 micrograms/mouse shows no toxicity and has no hemorrhagic activity (Siigur, *et al.*, 1991, Gasmi, *et al.*, 1991, Gasmi, *et al.*, 1997.). The other one beta-fibrinogenase of Levantine viper has weak caseinolytic activity and hydrolyzes glucagon at the sites Lys12-Tyr13, Arg17-Arg18 and Arg18-Ala19. In fibrinogen it cleaves B beta-chain first and later also the A alpha-chain. Lysine esters are not hydrolyzed (Siigur, *et al.*, 1991). Moreover, A separate Aα-, Bβ- fibrinogenolytic enzyme from Viper lebetina venom does not activate plasminogen or prothrombin but does degrade them both slightly (Gasmi *et al.*, 1991). The enzyme degrades the Aα-, Bβ- chain of fibrin rapidly, and also appears to degrade the γ- chain after prolonged (24 h) incubation. The enzyme inhibited platelet aggregation in human platelet rich plasma and has been used as a thrombolytic agent in rat venous thrombosis model (Gasmi *et al.*, 1997).

Table 2 Properties of snake venom β– chain Fibrinoginase.

properties / Venom source* ;	*C. atrox*	*C. atrox*	*C. atrox*	*T. gramineuse*	*T. mucrosquamatus*	*T. mucrosquamatus*
Common name	Protease II	Protease III	β -Fibrinoginase	β -Fibrinoginase	β -Fibrinoginase	P-1
Amino acid residues per molecule	284	198	N.D.	211	229	195
Molecular weight	31,000	24,000	22,900	25,000	26,000	23,000
pI	N.D.	N.D.	4.6	4.5	5.7	8.1
Carbohydrate content	+	-	N.D.	7 %	2 %	-
Enzymatic inhibitors	DFP,PMSF	DFP,PMSF	PMSF	PMSF	PMSF	EDTA
Inhibitor by human plasma proteinase inhibitor	α_2-M weak	None	N.D.	N.D.	Weak	N.D.
Optimal pH	8.5	10.5	N.D.	9.5	8.5	N.D.
Optimal temperature	50-65°C	50-65°C	N.D.	Heat stable	Heat stable	Heat labile
Hemorrhagic activity	N.D.	N.D.	N.D.	N.D.	-	-
Fibrinogenolytic	Bβ	Bβ	Bβ >Aα	Bβ >Aα	Bβ >>Aα	Bβ >Aα
Fibrinolytic activity	N.D.	N.D.	-	+(weak)	+(weak),(β, α polymer)	N.D.
References	(Pandya and Budzynski, 1984)	(Retzios and Markland, 1988; Pandya *et al.*, 1985)	(Sapru *et al.*., 1983)	(Ouyang and Huang, 1979)	(Ouyang *et al.*, 1977, 1979; Ouyang and Teng, 1976; Teng *et al.*, 1985)	(Sugihara *et al.*, 1985)

Table 2 Properties of snake venom β– chain Fibrinoginase (continued).

properties / Venom source* ;	*T. mucrosquamatus*				*T. mucrosquamatus*	*A. piscivorus piscivorus*
Common name	ME-1[b]	ME-2[b]	ME-3	ME-4	HF a	β -Fibrinoginase
Amino acid residues per molecule	249	271	300	263	131	306
Molecular weight	27,000	29,800	33,900	28, 500	15,000	33,500
pI	5.95	5.62	6.93	5.31	4.7	4.5
Carbohydrate content	0.4 %	0.2 %	6.4 %	1.3 %	N.D.	6 %
Enzymatic inhibitors	BZD	BZD	DFP	DFP	EDTA, PCMB	DFP
Inhibitor by human plasma proteinase inhibitor	N.D.	N.D.	N.D.	N.D.	N.D.	α,-M, ATIII
Optimal pH	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Optimal temperature	Heat stable	Heat stable	Heat stable	Heat stable	Heat labile	Heat stable
Hemorrhagic activity	-	-	-	-.	+	N.D.
Fibrinogenolytic	Bβ> Aα	Bβ>Aα	Aα= Bβ	Aα= Bβ	Bβ	Bβ
Fibrinolytic activity	+	+	+	+	N.D.	N.D.
References	(Sugihara *et al.*, 1986)	(Sugihara *et al.*, 1986)	(Sugihara *et al.*, 1986)	(Sugihara *et al.*, 1986)	(Nikai *et al.*, 1985)	(Nikai *et al.*, 1988)

Table 2 Properties of snake venom β– chain Fibrinoginase (continued).

properties / Venom source*	*T. mucrosquamatus*	*T. mucrosquamatus*	*V. lebetina*	*V. lebetina*
Common name	β -Fibrinoginase	FP	Fibrinoginase	β -Fibrinoginase
Molecular weight	28,000	22,000	26,000	52, 500
pI	N.D.	9.2	5.9	~3
Carbohydrate content	N.D.	+	5 %	23 %
Enzymatic inhibitors	PMSF	EDTA	EDTA	DFP, PMSF
Optimal temperature			Heat stable	Heat stable
Platelet activity	N.D.	N.D.	Inhibits ADP-induce PRP agg.	N.D.
Hemorrhagic activity	N.D.	+	-	N.D.
Fibrinogenolytic	Bβ> Aα	Bβ>Aα	Bβ>Aα	Bβ>Aα
Fibrinolytic activity	-	+	+	-
References	(Markland, 1998; Ouyang and Teng, 1976)	(Markland, 1998; Sapru *et al.*, 1983)	(Daoud *et al.*, 1988; Teng *et al.*, 1985; Daoud *et al.*, 1986; Markland, 1998)	(Nikai *et al.*, 1985; Ouyang *et al.*, 1979; Markland, 1998)

*Abbreviation: DFP = diisopropylfluorophosphate; PMSF = Phenylmethylsulfonyl fluoride; α_2-M = α_2-macroglobulin; α_1–AP = α_1- antiproteinase; ATII = antithrombin III; BZD = benzamidine; HF = hemorrhagic factor; + = activity is present; - = activity not detected; N.D. = not determined.

[a] Two chains, each with a molecular weight of 13,000. [b] May be identical to β- fibrinogenase of Ouyang and colleagues (Ouyang *et al.*, 1977; Ouyang and Teng, 1976; Ouyang and Huang, 1979; Teng *et al.*, 1985).

On the other hand, a previously reported of beta-fibrinogenase have a few researcher groups further studied in biochemical characterization and functional cloning. Hung *et al.* were studied *Trimeresurus mucrosquamatus* (Taiwan Habu), a snake species belonging to the Crotalidae family. it is a single-chain polypeptide with molecular weight of about 28,000. Its stability at high temperatures (> 90 degrees C) distinguished it from the previously reported venom fibrinogenases. N-Terminal sequence analysis revealed that it is similar to batroxobin and ancrod, which were shown to possess either fibrinogen-clotting or antithrombotic effect. Polymerase chain reaction (PCR) was employed to amplify cDNAs constructed from the poly(A)+RNA of fresh venom glands of the same snake species to facilitate the cloning and sequencing of this important fibrinogenase. Sequencing several positive clones corresponding to the coding sequence of the enzyme revealed the existence of a family of novel thrombin-like fibrinogenases in the Taiwan habu, which are heat-stable and may be useful as strong antithrombotic agents(B6). In addition Hung and Chiou(2001) were studied fibrinogenolytic proteases isolated from the snake venom of Taiwan Habu: Serine Proteases with Kallikrein-like and Angiotensin-Degrading Activities. Which are containing two venom proteases with fibrinogenolytic activity, one major crotalid snake species in Taiwan. The purified enzymes showed a strong B β -fibrinogenolytic activity, cleaving β -chain of fibrinogen molecules specifically. They also showed strong kallikrein-like activity *in vitro,* releasing bradykinin from kininogen. The purified enzymes did not coagulate human plasma, yet decreasing fibrinogen levels in plasma and prolonging bleeding without formation of fibrin clots, indicating that both proteases have specificities different from thrombin and thrombin-like proteases of snake venom reported previously. They also exhibit amidase activity against *N*-benzoyl-Pro-Phe-Arg-*p*-nitroanilide, which is a specific synthetic substrate for kallikrein-like proteases. Their stability at high temperatures was examined and found to be more stable when compared with ancrod and thrombin. Intravenous injection of either protease was shown to lower blood pressure in experimental rats. Most noteworthy is the observation that the proteases can cleave angiotensin I and release bradykinin from plasma kininogen *in vitro,* which is a strong vasodilator and probably responsible for the *in vivo* hypotensive effect of these venom proteases (Hung, and Chiou, 2001).

Other one is mucrosobin was cloned and functional expression of the protein, a beta-fibrinogenase of *Trimeresurus mucrosquamatus* (Taiwan Habu) by Guo *et al.*. The full-length cDNA of mucrosobin was assembled by oligonucleotide screening and 5'-rapid amplification of cDNA ends. The amino acid sequence deduced from the cDNA consists of 257 amino acid residues with a putative signal peptide of 24 residues. It is highly homologous to the other thrombin-like enzymes (batroxobin, mucofirase, and calobin), suggesting that it is a serine proteinase with a conserved catalytic triad of His(41), Asp(84) and Ser(179) in the deduced form of mucrosobin protein. Northern blot analysis revealed that the mucrosobin gene encodes an mRNA of 1.5 kb and suggested a tissue-specific expression in the venomous gland. In an effort to study the biological property of mocrosobin, we have expressed the 28-kDa protein as inclusion bodies in Escherichia coli. For analyzing enzymatic activity, the inclusion bodies were solubilized and the recombinant protein was refolded with a two-step dialysis protocol. The refolded recombinant protein exhibited a specific beta-fibrinogenolytic activity. This study offers a possibility of using genetic engineering to acquire a functional snake venom protein with therapeutic potential (B7).

In *Agkistrodon shedaoenthesis* Zhao was studied by Mang JIAO *et al.*. Shedaoenase; The N-terminal sequence of shedaoenase was determined, and its full-length cDNA encoding a protein of 238 amino acid residues was cloned by reverse transcription-polymerase chain reaction from the total mRNA extracted from the snake venom gland. The primary structure of shedaoenase has the typical character of the snake venom serine proteases. Based on the sequence similarity to other venom proteases, the reactive sites of the catalytic triad in shedaoenase are presumed to be His43, Asp88 and Ser194, corresponding to those of His40, Asp84 and Ser176 in trypsin. The sequences around these three amino acid residues were found to be highly homologous to other serine proteases. Furthermore, most of the snake venom serine proteases belong to the trypsin-like serine protease family. The bottom of the S1 pocket of trypsin-like serine proteinases is highly conserved with an Asp, which forms a canonical ion pair interaction with the positively charged side chain of the P1 residue

of the substrate molecule. This Asp is at the position six residues prior to the active serine. Shedaoenase contains six disulfide bridges, as do most of the snake venom serine proteases. These disulfide bridges are assigned from the sequence similarity with trypsin, as follows: Cys7-Cys141, Cys28-Cys44, Cys76-Cys236, Cys119-Cys190, Cys151-Cys168, and Cys180-Cys206. The molecular mass of shedaoenase, based on the deduced amino acid sequence, is approximately 26.4 kDa, whereas the apparent molecular mass according to the result of SDS-PAGE was estimated to be 36 kDa. This discrepancy may be due to the alteration of electrophoresis behavior caused by the oligosaccharide chains linked to shedaoenase. This presumption was corroborated by the sequence analysis. Shedaoenase has two potential Nglycosylation sites at Asn80 and Asn100. The Asn80-Tyr81- Thr82 and the Asn100-Ser101-Thr102 residues might be the NX- T(S) motif, known to be a potential N-glycosylation site linked to asparagines (Jiao, *et al.*, 2005).

Biochemical characterization of beta-fibrinogenase from the venom of *Vipera lebetina* [*Macrovipera lebetina* venom (Levantine viper)] were studied by Siigur *et al.*. This enzyme comprised neutral sugar, hexosamines 8.3%, Hexose 23.0% and Sialic acid 1.0%. β – fibrinogenase is a trypical arginine esterase which dose not attack protein substrate such as casein and insulin. Amidase activity of β –fibrinogenase is limited with plasma kallikrein specific substrate Pro-Phe-Arg-MCA the other peptide are weakly hydrolyzed. Preference of kallikrein substrate by β –fibrinogenase may point to the multifunctional activity of this protsin. There are some multifunctional enzyme isolated from snake venom-kinin-releasing and fibrinogen-clotting crotalase from Crotalus adamanteus venom (B16). Amino acid sequences of β -fibrinogenase (VLBF) from *Vipera lebetina* venom have been deduced from the cDNA sequences encoding the enzymes. The mature protein sequences of 233 amino acids (VLBF) exhibit significant similarity with other snake venom serine proteinases. β - fibrinogenase was containing the catalytic triad His57, Asp102, Ser195, and twelve conserved cysteines forming six disulfide bridges. Unlike typical trypsin-like serine proteinases, they lack the third aspartate, Asp189 which is replaced by Gly189.VLBF is a typical representative

of arginine esterases – β -fibrinogenases. The VLBF gene includes a 5'-UTR – nucleotides 1-180, a proenzyme coding region (nt 181-252), a mature enzyme coding region (nt 253-954), and a 3'-UTR (nt 955-1580). The highest identity (87-88%) occurs with the cDNAs of *T. gramineus* and *T. flavoviridis*, TLf3, TLf2, TLg2a (11). The open reading frame for VLBF encodes a protein comprising 257 amino acids: 24 amino acids of prepropeptide followed by 233 amino acid mature protein. The calculated molecular mass for VLBF is 25580 which is considerably lower than that of the native enzyme (42.2 kDa). The difference is caused by heavy glycosylation of the native enzyme. Taking 2.5- 3.0 kDa for the carbohydrate chain increment, VLBF should contain 6 glycosyl chains. Actually the deduced protein sequence exhibits 5 putative N-glycosylation sites at the residues Asn36, Asn94, Asn109, Asn147, Asn245. Potential Oglycosylation site is located at the position Ser125. The sequence alignment with some other snake venom serine proteinases . The identity is highest between α-fibrinogenase *Macrovipera lebetina* (VLAF) and VLBF themselves –77%. The rate of identity with other snake venom serine esterases covers the range of 60-73%.

By the way, Most of the snake venom serine proteases are very similar to each other in amino acid sequence, which implies that they might have evolved from the same precursor protein and possibly adapted to each target protein of the snake's prey. In spite of their high homology in primary structure and tertiary structure, the snake venom serine proteases show various substrate specificities. This may be due to some refined difference of the structure at the substrate-binding site. Some surface loops surrounding the extended substrate-binding site may contribute to this diversity (Maroun *et al.*, 2001; Guillin *et al.*, 1995; Zhu *et al.*, 2005). So that the venom fibrinogenolytic serine proteinase offers an interesting dilemma with respect to enzyme classification, much as the hemorrhagic and fibrinolytic metalloproteinases do. These proteinases, as well as the venom plasminogen activator, share extensive sequence homology with the thrombin-like venom serine proteinases ancrod (Burkhart *et al.*, 1992; Nolan *et al.*, 1975), batroxobin (Stocker and Barlow, 1976; Itoh *et al.*, 1987;) and crotalase (Markland and Damus, 1971; Markland, 1976), (**Figure 3**) and

with other serine proteinases such as the kallikrein-like enzyme from *C. atrox* (Bjarnason *et al.*, 1983) and the protein C activator from *A. c. contortrix* (Stocker *et al.*, 1987) venoms. Clearly there are subtle differences between these homologous enzymes that determine whether they exhibit fibrinogenolytic, plasminogen activator, thrombin-like, or other specific enzymatic activities.

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                1         10         20         30         40 *       5(
Ancrod          VIGGDECNINEHRFLVAVYEGTNWTFICGGVLIHPEWVITAEHCARRRMN
Batroxobin      VIGGDECDINEHPFLAFMYY SPRYF CGMTLINQEWVLTAAHCNRRFMR
Crotalase       VIGGDECNINEHRFLVALYDYWSQLFLCGGTLINNEWVLTAAHCDRTHIL
TSV-PA          VFGGDECNINEHRSLVVLFNSNGFL  CGGTLINQDWVVTAAHCDSNNFQ
Tm-Vig          VIGGDECNINEHPFLVLVYYDDYQ   CGGTLLNEEWVLTAAHCNGKDME

                          60         70         80         * 90
Ancrod          LVFGMHRKSEKFDDEQERYPKKRYFIRCNKTRTSWD  EDIMLIRLNKPV
Batroxobin      IHLGKHAGSVANYDEVVRYPKEK FI CPNKKKNVITDKDIMLIRLDRPV
Crotalase       IYVGVHDRSVQFDKEQRRFPKEKYFFDCSNNFTKWD  KDIMLIRLNKPV
TSV-PA          LLFGVH  SKKILNEDEQTRDPKEKFFCPNRKKDDEVDKDIMLIKLDSSV
TM-VIG          IYLGVH  SKKVPNKDVQRRVPKEKFFCDSSKTYTKWNKDINLIRLDRPV

                 100        110        120        130        140
Ancrod          NNSEHIAPLSLPSNPPIVGSDCRVMGWGSINRRIDVLS DEPRCANINLH
Batroxobin      KNSEHIAPLSLPSNPPSVGSVCRIMGWGAITTS EDTYPDVPHCANINLF
Crotalase       SYSEHIAPLSLPSSPPIVGSVCRAMGWGQTTSP QETLPDVPHCANINLL
TSV-PA          SNSEHIAPLSLPSSPPSVGSVCRIMGWGKTI PTKEIYPDVPHCANINIL
TM-VIG          RKSAHIAPLSLPSSPPSVGSVCRVMGWGTITSP QETYPDVPHCANINLL

                  150        160        170        *          190
Ancrod          NFTMCHGLFRKMPKKGRV  LCAGDLRGRRDSCNSDSGGPLICNEELHGI
Batroxobin      NNTVCREAYNGLPAKT    LCAGVLQGGIDTCGGDSGGPLICNGQFQGI
Crotalase       DYEVCRTAHPQFRLPATSRTLCAGVLEGGIDTCNRDSGGPLICNGQFQGI
TSV-PA          DHAVCRTAYSWRQVANT   TLCAGILQGGRDTCHFDSGGPLICNGIFQGI
TM-VIG          DYEVCRAAYAGLPATSR   TLCAGILEGGKDSCVGDSGGPLICNGQFQGI

                     200        210        220        230
Ancrod          VARGPNPCAQPNKPALYTSIYDYRDWVNNVIAGNAT  CSP
Batroxobin      LSWGSDPCAEPRKPAFYTKVFDYLPWIQSIIAGNKTATC P
Crotalase       VFWGPDPCAQPDKPGLYTKVFDHLDWIQSIIAGEKTVNC P
TSV-PA          VSWGGHPCGQPGEPGVYTKVFDYLDWIKSIIAGNKDATCPP
TM-VIG          VSWGGDPCAQPREPGVCTNVFDHLVWIKGIIAGNTDVTC PL
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Figure 3 Alignment of sequences of ancrod (defibrinogenating enzyme from *Calloselasma rhodostoma* venom; 234 amino acids) (Burkhart *et al.*,1992), batroxobin (defibrinogenating enzyme from *Bothrops atrox* venom; 231 amino acids) (Itoh *et al.*, 1987), crotalase (defibrinogenating enzyme from *Crotalus adamanteus* venom; 237 amino acids) (Henschen-Edman *et al.*, 1999), TSV-PA (plasminogen activator from *T. stejnegeri* venom; 234 amino acids) (Zhang *et al.*, 1995), and TM-VIG (b-chain fibrinogenase from *Trimeresurus mucrosquamatus* venom; 233 amino acids) (Hung *et al.*,1994). Sequences of the proteases were aligned by the positioning of the 12 cysteine residues. Spaces were inserted as needed for proper alignment. Catalytic triad residues histidine 43, aspartic acid 88, and serine 182 (ancrod numbering) shown by *. Residues underlined are identical in all three enzymes (92/231 or ~39.8%).