Snake Venom Proteinases as Tools in Hemostasis Studies: Structure-Function Relationship of a Plasminogen Activator Purified from *Trimeresurus stejnegeri* Venom

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Key Words

Snake venom · Proteinase · Plasminogen activator · Structure-function · Haemostasis

Abstract

Snake venom serine proteinases affect many steps of the blood coagulation cascade. Each of them usually acts selectively on one coagulation factor. They are therefore potentially useful components to study the mechanisms of action, the regulation and the structure-function relationships of human serine proteinase coagulation factors. This strategy is illustrated for a plasminogen activator purified from *Trimeresurus stejnegeri* venom.

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Serine Proteinase Family

Serine proteinases are found in microorganisms [1], plants [2] and numerous animals [3, 4]. These enzymes share many biochemical and structural properties, such as a catalytic triad (Ser¹⁹⁵, His⁵⁷, Asp¹⁰²), the main criterion for classification of a protein as a serine proteinase. They exhibit structural differences which can be used to classify this family into three subgroups: trypsin-like, subtilisin-like and carboxypeptidase-like serine proteinase [3].

Human Serine Proteinases Involved in Hemostasis

Human serine proteinases have diverse functions and may be involved in digestion, complement activation, cellular differentiation and hemostasis [3]. In particular, human trypsin-like serine proteinases are involved in

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0301-0147/01/0316-0133\$17.50/0

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hemostasis, with roles in platelet aggregation, coagulation and fibrinolysis (fig. 1). Their activity is regulated by various families of inhibitors, notably the serine proteases inhibitors (serpins), which slowly form irreversible complexes with the protease.

Human serine proteinases active in hemostasis generally contain at least a catalytic domain structurally similar to trypsin. Some also possess, at their N-terminal end, one or several additional domains (kringle, apple, finger or epidermal growth factor domains) that are involved in protease interactions with cofactors and cellular receptors. Alignments of the amino acid sequences of their catalytic domain show insertions at the variable regions, which are involved in the interaction of the serine proteinases with their macromolecular substrates, and/or specific inhibitors, as demonstrated by site-directed mutagenesis studies [5–8].

The crystal structures of more than twenty trypsin-like serine proteinases have been determined. They include those of bovine chymotrypsin [9], bovine trypsin [10], porcine kallikrein [11], human thrombin [12] and the catalytic domains of human factor X [13] and human tissue-type plasminogen activator (t-PA) [14]. The overall structure of serine proteinases is highly conserved in all members of the family [15, 16], since they are all folded into two six-stranded β -barrels, separated by the catalytic residues, and a C-terminal helical segment. In trypsin-like serine proteinases, the S_1 pocket is highly conserved, particularly the aspartate residue at position 189, which forms a canonical ion pair interaction with the positively charged side chain of the P_1 residue of the substrate molecule [17].

However, there are still major unanswered questions concerning the structure of the human serine proteinases involved in hemostasis. These questions mainly concern the dynamics of activation of the zymogen in potent

enzymes, inhibition by physiological inhibitors and the molecular effects of these enzymes on their protein targets. To characterize the structural elements involved in target molecule recognition, snake venom trypsin-like serine proteases provide excellent models.

Snake Venom Serine Proteinases Affecting Hemostasis

Serine proteinases are very abundant in Viperidae and Crotalidae venoms in which they may account for 20% of the total protein content. They are not lethal by themselves, but they contribute to the toxic effect of the venom when associated with other venom proteins. They selectively affect many steps in the blood coagulation cascade by the activation or the inhibition of specific blood factors involved in platelet aggregation, coagulation or fibrinolysis (fig. 1) [18–20].

Various snake venom serine proteinases have a platelet-aggregating activity. The mechanism of action of MSP1 (*Bothrops moojeni*), cerastobin (*Cerastes vipera*), cerastocytin and cerastotin (*Cerastes cerastes*) on platelet activation is unknown [21, 22], but it has been reported that thrombocytin (*Bothrops atrox*) and PA-Bj (*Bothrops jararaca*) activate proteinase-activated receptors 1 and 4 of thrombin (PAR-1 and PAR-4) in human blood platelets [23, 24]. Other snake venom serine proteinases affect coagulation factors. A coagulant factor from Russell's viper venom (RVV-V), selectively activates factor V, converting it to FVa by cleaving a single peptide bond [25]. An anticoagulant enzyme from *Agkistrodon contortrix contortrix* venom activates protein C (ACC-C) [26]. The resulting activated protein C then degrades factor Va and factor VIIIa, resulting in an anticoagulant effect. Many other Viperidae serine proteinases convert fibrinogen into fibrin by cleaving fibrinopeptides A and/or B [27–29].

Fig. 1. Physiological processes of hemostasis. Action of various snake venom serine proteinases on the three pathways of hemostasis: platelet aggregation (**a**), coagulation (**b**) and fibrinolysis (**c**). Snake venom serine proteinases are in bold.

As this activity resembles the most well-known activity of thrombin, these venom components are generally termed 'thrombin-like' enzymes. However, the fibrin monomers generated by venom thrombin-like enzymes undergo limited polymerization, because only one fibrinopeptide is generally cleaved and because these enzymes do not activate factor XIII, to generate factor XIIIa. The clot is stabilized as the factor XIIIa catalyzed cross-linking of fibrin monomers. Many snake venom enzymes share one or several, but not all, the activities of human thrombin. They are therefore interesting tools for investigating the various activities of this multifunctional enzyme. On the other hand, *Trimeresurus stejnegeri* venom plasminogen activator (TSV-PA) is the first serine proteinase from snake venom reported to activate fibrinolysis [30].

Snake venom serine proteinases like the human serine proteinases affecting hemostasis belong to the trypsin-like subfamily. Alignments of the sequences of these proteinases with the catalytic domains of human serine proteinases highlight the presence of restricted variable regions in the snake venom enzymes (fig. 2). The restriction of these regions in snake venom serine proteinases probably accounts for their resistance to physiological inhibitors and raises interesting questions concerning their substrate specificity. They also display high levels of sequence identity, despite having different but specific physiological properties.

The crystallographic structure of a snake venom serine proteinase (TSV-PA) was determined [31]. Detailed analysis of the three-dimensional structure of TSV-PA indicates that access to the active site is restricted to the west by a loop segment. This loop has one more amino acid in TSV-PA than in trypsin, at position 218, and a proline is present in a *cis* conformation at position 219. The main

chain of this loop has a conformation similar to that of the ancestral molecule, glandular kallikrein, which shows 36.5% identity to TSV-PA at the amino acid sequence level. The kallikrein loop also has an additional amino acid, at position 218, and a *cis* Pro²¹⁹ [11], consistent with the proposed evolution of TSV-PA from glandular kallikrein [32–34].

Structure-Function Study of TSV-PA: An Approach to New Thrombolytic Agents

Since thrombotic disorders remain a major cause of morbidity and mortality in many countries, studies on the fibrinolytic system, which uniquely counterbalances the blood coagulation cascade, have called for intense research efforts [35–37]. The rate-limiting step in fibrinolysis is catalyzed by t-PA, a member of the serine proteinase family which converts plasminogen into an active proteinase plasmin [37]. The specificity of t-PA for plasminogen, which has been attributed to its simultaneous binding to fibrin through its kringle domains [38], is in fact an inherent property of its protease domain since it is maintained in the absence of fibrin [39, 40]. On the other hand, recent structural investigations confirm the close similarity of the t-PA proteinase

Fig. 2. Sequence alignments. The amino acid sequences of batroxobin [43], Ancrod [44], TSV-PA [30], RVV-V [45], porcine glandular kallikrein [11], human t-PA [14] and bovine chymotrypsin [46] were aligned according to their topological equivalence. The numbering refers to chymotrypsinogen. Residues of the catalytic triad are marked by stars. Particularly close sequence homologies between all seven proteins are given in pale gray boxes, where identities are typed in dark gray. The figure was prepared using the Clustal W program.

	16	20	30	40	50	60
Batroxobin	VIGS	DECDINEHPFLAFMYISP	-----	RYFCGMT	INQEWLTAHCHNRR	-----FMRI
Ancrod	VIGS	DECNINEHRFLALVYAN	-----	GSLCGGT	INQEWLTAHCHDRG	-----NMRI
TSV-PA	VFGS	DECNINEHRSLVFLFNSN	-----	GFLCGGT	INQDVVVTAAHCDN	-----NFQL
RVV-V	VVG	DECNINEHPFLVALYTSTS	----	STIHCGGAL	INREWLTAHCHDRR	-----NIRI
Kallikrein	IIG	RECEKNSHPWQVAIYHYS	-----	SFQCGGV	LVNPKVLTAAHCKND	-----NYEV
Human t-PA	IKG	SLFADIASHPWQAATFAKHRRSPGERFLCGGIL	ISSC	WILSAHCFQERFP	PHHLTV	
Chymotrypsin	IVN	EEAVPGSWPWQVSLQDKTG	----	FHFCGGS	INENWVVTAAHCGVT	-----TSDVV
					*	
	70	80	90	100	110	
Batroxobin	HLG	KHAGSVANYDEVVRYPKEKFCP	--	NKKKN-VITDKDIM	IRIDRPVK	-----NSEHI
Ancrod	YLG	MHNKLVNKDALRRFPKEKYFCL	--	NTRND-TIWDKDIM	IRINRPVR	-----NSAHI
TSV-PA	LFG	VHKKILNEDEQTRDPKEKFFCP	--	NRKKD-DEVDKDIM	IKLDSSVS	-----NSEHI
RVV-V	KLG	MHSKNIRNEDEQIRVPRGKYFCL	--	NTKFP-NGLDKDIM	IRLRPVT	-----YSTHI
Kallikrein	WLG	RHNLFENENTAQFFGVTA	DFPHPGFNLSADGKDYS	HDLM	LRLQSPAK	-----ITDAV
Human t-PA	ILG	RTYRVVPGEQKFEVEKYIVHK	----	EFDDTYDNDIAL	LQLKSDSSRCAQESSV	
Chymotrypsin	VAG	EFDQGSSEKIQKLKIAKVPKNS	----	KYNLSLTINNDIT	LKLSAAS	-----FSQTV
					*	
	120	130	140	150	160	170
Batroxobin	APLS	SPSNPPSVG--SV	RIMGWCAITTS	ED--	TYPDVPHCANINL	FNNTVORE-AYNGL
Ancrod	APLS	SPSNPPSVG--SV	RIMGWCTITSPNA--	TLPDVPHCANIN	ILDYAVQA-AYKG-	
TSV-PA	APLS	SPSPSVG--SV	RIMGWKTIPTKE--	IYPDVPHCANIN	ILDHAVOR	RT-AYSWR
RVV-V	APVS	SPSRSGVG--SR	RIMGWKISTTED--	TYPDVPHCTNIF	IVKHKWEP-LYPWV	
Kallikrein	KVLE	LPTQPELG--ST	EASGWSIEPGDB	FEFDEIQCVQLTL	LQNTFAB-AHPB-	
Human t-PA	RTVC	LPADLQLPDWTE	ELSELYKKHEALSP--	FYSERLKEAHVRL	YPSSRRTSQHLLNR	
Chymotrypsin	SAVCL	SPASDDFAAGTT	VTTFWGLTRYTNA--	NTPDRLQQASL	PLLSNTNK--KYWGT	
					*	
	180	190	200	210	220	
Batroxobin	--	PAKTLCAQVLQGG-----	IDTCGGDSGGPLI	ENG----	QFQSILSWSDP	CAEPRKP
Ancrod	--	AATTLCAQILEGG-----	KDTCKGDSGGPLI	ENG----	QFQSILSVGNP	CAQPRKP
TSV-PA	QVANT	TLCAGILQGG-----	RDTCHFDSGGPLI	ENG----	IFQSIVSWGH	PCGQPGEE
RVV-V	PADS	RTLCAQILKGG-----	RDTCHGDSGGPLI	ENG----	QIQSIVAGSE	PCGQHLKE
Kallikrein	KVTES	MLCAQYLPGG-----	KDTCMGDSGGPLI	ENG----	MWQSITSWHT	POGSANKP
Human t-PA	TVTDN	MMLCAGDTRSGGPQANL	HDAQGDSGGPLV	CLNDGRMTLV	GIISWELG-CGQK	DVNE
Chymotrypsin	KIKDA	MICAGASG-----	VSSCMGDSGGPLV	CKKNGAWTLV	GIISWELGSS	TCS-TSTP
					*	
	230	240				
Batroxobin	AFV	TKVFDYLPWIIQSI	IAGNKTATCP-			
Ancrod	GI	TKVFDYTDWIIQSI	ISGNTDATCPP			
TSV-PA	GV	TKVFDYLDWIKSI	IAGNKDATCPP			
RVV-V	AV	TKVFDYNNWIIQNI	IAGNRTVTCPP			
Kallikrein	SI	TKLIFYLDWINDT	TENP-----			
Human t-PA	GV	TKVTNYLDWIRD	NMRP-----			
Chymotrypsin	GV	ARVTALVNVVQQT	LAAN-----			

domain to that of nonspecific proteinases such as trypsin [14, 16]. Nevertheless, the molecular basis for the specificity of t-PA for plasminogen remains poorly understood. TSV-PA, which like t-PA, selectively converts plasminogen into plasmin by cleavage of the peptide bond Arg⁵⁶¹-Val⁵⁶², is a useful structural template for the identification of the site of interactions of these proteins with plasminogen and the plasminogen activator inhibitor-1 (PAI-1).

Although TSV-PA shares only 23% sequence identity with the catalytic domain of t-PA, its tridimensional structure demonstrates a close structural similarity to t-PA [31]. Close to its active site, TSV-PA possesses the 99-loop which projects from the molecular surface and forms a highly charged rim (Lys⁹⁴-Lys⁹⁵-Asp⁹⁶-Asp⁹⁷-Glu⁹⁸-Val⁹⁹). A salt bridge between Asp⁹⁷ and Arg¹⁷⁴ stabilizes this loop and occludes the S₄ binding pocket. The replacement of the sequence Asp⁹⁶-Asp⁹⁷-Glu⁹⁸ to Asn-Val-Ile resulted in a loss of plasminogenolytic activity, indicating the key role this sequence plays in substrate recognition by the enzyme. Separate replacement of each of these negatively charged residues at the site indicated that Asp⁹⁷ was the most critical residue. The Asp⁹⁷-Val point mutation resulted in a 125-fold decrease in TSV-PA activity for plasminogen activation [41]. Mutational studies around the segment 172–176, however, have shown little or no effect on the plasminogenolytic activity of TSV-PA. It is therefore reasonable to assume that Asp⁹⁷ changes its conformation upon complex formation and participates in secondary contacts with plasminogen. The conservation of Asp⁹⁷ in urokinase-type PA (u-PA) and t-PA is noteworthy. It is likely that Asp⁹⁷ of t-PA and u-PA have a similar role in plasminogen recognition.

The ability of a serpin to neutralize a given serine proteinase appears to depend both

upon the sequence of its reactive center loop and the contribution of one (or more) secondary binding site(s). TSV-PA escapes some serpins by the use of at least two molecular mechanisms: the first involves Phe¹⁹³, a residue of the catalytic groove, the second originates from the absence of secondary binding sites as positively charged residues in the variable region-1 (VR1) of TSV-PA. While all other trypsin-like proteinases have a glycine at position 193 in the chymotrypsin numbering system, TSV-PA has a phenylalanine, and this phenylalanine restricts access to the S'2 pocket. The TSV-PA mutant (F193G), in which Phe¹⁹³ had been replaced by a glycine, was inhibited by PAI-1, α_2 -antiplasmin and α_1 -antitrypsin with association rate constant (kon) values at least twenty-fold higher than for the wild-type enzyme. On the other hand, substitution of the VR1 of TSV-PA for either that of t-PA or u-PA also increased the kon value for the inhibition by PAI-1 over hundred times. Mutations were additive, for the inhibition by PAI-1: the double TSV-PA variant F193G/t-PA (VR1) was inhibited with a kon value 4 orders of magnitude higher than the wild-type enzyme. We conclude that these two motifs of TSV-PA fully account for its resistance to PAI-1. This opens the doors to therapeutic improvement of presently used fibrinolytic agents, since construction of a t-PA variant containing a Phe at position 193 could give a new thrombolytic agent with a longer half-life since it resists longer to PAI-1 not only in systemic circulation but also at the clot site [42]. Moreover, the TSV-PA variant F193G is a good candidate for the crystallographic resolution of a complex between a proteinase and a serpin since it produces SDS-stable complexes with PAI-1, α_2 -AP and α_1 -AT. Indeed, the accession of this kind of complex could improve our understanding of the inhibitory mechanism of serpins.

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