Vipera lebetina Venom Contains All Types of Snake Venom Metalloproteases

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Key Words

Fibrino(geno)lytic enzymes · Lebetase ·
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Abstract

Snake venoms contain four classes of metalloproteases that all have a typical zinc-chelating sequence (HEXXH-GXXH). N-terminal sequences and internal sequences of different purified metalloproteases were determined using Edman sequencing and LC MS/MS technique. Oligonucleotides were designed and used as primers for cDNA cloning from *Vipera lebetina* venom gland cDNA library. We found that isoforms of fibrinolytic enzyme lebetase Le-4 and Le-3 are synthesized in different way: Le-4 is synthesized as P-I type metalloprotease, Le-3 is synthesized with disintegrin-like domain as P-II type protease and processed post-translationally. An endothelial cell apoptosis-inducing heterodimeric glycosylated metalloprotease, *V. lebetina* apoptosis-inducing protease (VLAIP), belongs to P-III type containing metalloprotease, disintegrin-like and cysteine-rich domains. All these enzymes hydrolyze the A α -chain and more slowly the B β -chain of fibrinogen. Treatment of HUVEC cells with VLAIP induces changes in the attachment of cells to the substrate and causes apoptosis. *V. lebetina* venom contains also P-IV type-specific coagulant factor X activator

(VLFXA) that cleaves the Arg⁵²-Ile⁵³ bond in the heavy chain of human factor X. VLFXA is a glycoprotein composed of a heavy chain and two C-type lectin-like light chains linked by disulfide bonds. The heavy and light chains of VLFXA are synthesized from different genes.

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Introduction

Snake venoms, particularly those belonging to the Crotalidae and Viperidae families, contain a wide variety of proteases that induce alterations in the blood coagulation cascade [1–7]. Proteases so far isolated from snake venoms are usually divided into two groups: (i) metalloproteases which need Ca²⁺ or Zn²⁺ (or both) for their hydrolytic activity and are inhibited by metal chelating agents (factor X activator, prothrombin activator, $\alpha(\beta)$ -fibrinogenases, hemorrhagic proteases, and apoptosis-inducing proteases; and (ii) serine proteases (factor V activator, protein C activator, plasminogen activator, kinin-releasing and thrombin-like enzymes, β -fibrinogenases, some factor X activators, and prothrombin activators). Highly toxic hemorrhagic proteases degrade mammalian tissue proteins in a non-specific manner. A number of venom proteases cleave plasma proteins in a relatively specific manner.

Zinc metalloproteases are widely occurring and participate in a number of important biological, physiological, and pathophysiological processes (hemorrhage, fertilization, thrombolysis, cancer metastasis, etc.). Because of the possible therapeutic and diagnostic role of snake venom proteases, these enzymes merit further investigation. *Vipera lebetina* (Levantine viper) is a snake found in South-East parts of Europe, in South-West Asia and in North-West Africa. *Vipera lebetina* venom contains various metalloproteases, which act on coagulation through both pro- and anticoagulant mechanisms and also proteases acting on extracellular membranes causing hemorrhage.

Due to potential use of snake venom proteases as diagnostic, thrombolytic, apoptosis-inducing agents, it is important to know their structure-function relationship. In this short review, we show that *V. lebetina* venom contains representatives of all four classes of reprotlysins.

Snake Venom Metalloproteases – Reprotlysins

The term reprotysin was proposed by Bjarnason and Fox [8] to stress ‘the reptilian and reproductive’ origin of these proteases.

Viperid and crotalid snake venoms contain a number of metalloproteases that are divided into P-I to P-IV structural classes [9, 10]. P-I enzymes contain metalloprotease domain only, P-II enzymes include metalloprotease and disintegrin(-like) domains, P-III proteins contain, in addition, cysteine-rich domain, and P-IV proteins comprise metalloprotease, disintegrin-like, cysteine-rich, and lectin-like domains. These snake venom metalloproteases are synthesized in zymogen form from corresponding mRNAs. The P-I class metalloproteases are synthesized from N-I class polynucleotides encoding signal peptide, propeptide, and metalloprotease domains, or from N-II class polynucleotides encoding after metalloprotease domain a disintegrin(-like) domain which will be post-translationally processed to metalloprotease and disintegrin. In some cases, N-II class will give P-II proteins as well. P-III proteases are synthesized from N-III polynucleotide class encoding signal peptide, propeptide, metalloprotease, disintegrin-like, and cysteine-rich domains. P-IV class proteases are synthesized as P-III ones (heavy chain), the light chain(s) are synthesized from individual gene and connected with P-III protein post-translationally [11]. There is no evidence for N-IV polynucleotide class. Metalloprotease domains of snake venom metalloproteases have homologous se-

quences and typical active site structure for reprotlysins HEXHXXGXXH.

Recently, a slightly modified classification scheme of the snake venom metalloproteases has been developed by Fox and Serrano [12]. In this version, they have noted that there are subclasses within the P-II and P-III that reflect the potential for proteolytic processing and for the formation of dimeric structures. Metalloproteases isolated from snake venoms are members of the reprotysin subfamily that also includes A Disintegrin and Metalloprotease (ADAM). Reprotlysins and ADAMs have similar domain structure but ADAMs additionally contain an epidermal growth factor-like domain, a transmembrane region and cytoplasmic tail [13].

P-I and P-II Class Lebetase Isoforms

Lebetase (EC 3.4.24; M12.164) from *Vipera lebetina* venom is a non-glycosylated direct-acting fibrinolytic metalloprotease, as it acts via direct cleavage of fibrin, not by plasminogen activation [14, 15].

Lebetase degrades fibrinogen α and β chains and the cleavage products arising from the different isoforms are identical. Lebetase is inhibited with dithiothreitol and EDTA. It contains 1 mol of Zn per mol of protein. Lebetase has at least four isoforms with pIs in the interval of 4.6–5.6. Lebetase isoforms have different pH stability [16]. The isoform with pI of 5.3 and molecular mass of 22,890 (corrected value) has been cloned and sequenced [17]. It is synthesized from N-II class mRNA comprising signal peptide, proenzyme, mature metalloprotease, and disintegrin-like domains in the translated sequence. The enzyme is synthesized in venom glands as a P-II type protease as based on the modified classification scheme of the snake venom metalloproteases [12]. The post-translational processing separates the metalloprotease and disintegrin-like domains. The disintegrin-like domain exists as a monomer in the heterodimeric disintegrin, like VLO5

Fig. 1. Comparison of deduced amino acid sequences of *V. lebetina* venom metalloproteases; VLAIP-A(AY835996), VLAIP-B(AY835997)-heterodimeric apoptosis-inducing protease [21]; LFXA-HC(AY339162)-factor X activator heavy chain [11]; Le-3(X97894)-isoform of fibrinolytic enzyme lebetase [17]; Le-4(AY987816)-isoform of fibrinolytic enzyme lebetase. Amino acid residues that are identical to those of VLAIP-A are indicated by dots, and caps are indicated by dashes.

VLAIP-A	1	MMQVLLVTISLAVFPYQGSSIIILESGNVNDYEVVYPQKV	TAMPKGA	VQPEQKYEDAMQY	60				
VLAIP-B	1	C.....	I..L...	IQ.....IK.	60				
VLFXA-HC	1		I..L..EE..Q.....	T...	60				
Le-3	1	.I.....C.....	KT.K.....N..A..GL.....	K...T...	60				
Le-4		signal prepropeptide							
VLAIP-A	61	EFKVKGE	PVVLLLEKNKDLFSE	DYSETHYSPDGREITT	NPPVEDHCYYHGRIQN	DADSSA	120		
VLAIP-B	61	N.K....	H....G.....	T.....I.....	T.	120			
VLFXA-HC	61	..E.N.....	H.....R.....	T..K...Q.....	Y...	120			
Le-3	61	..E.N.....	H....RG...K.....	A.....	T.	120			
Le-4									
VLAIP-A	121	SISACNGLK	GHFMLQGETY	LIEPLKLPDSEAH	AVYKYENVEKEDE	APKMG	CVTQTNWESD	180	
VLAIP-B	121	K....	M.....RI.....	I.....I.....				180	
VLFXA-HC	121	K.....	I.....					180	
Le-3	121	Y.T.R.....		I.....	A..			180	
Le-4									
VLAIP-A	181	EPIKKASQL	NLTPEQRRYLNSPKYIKL	VIVADYIMFLKYGRSLITIR	TRIYEIVN	ILNVI		240	
VLAIP-B	181	-.....	T..K.K..VE.....	W..D...S.....	T...			239	
VLFXA-HC	181	VA.SAK	KFHKT--F.E...	V.HRVVK..DSA--	ATN.K....	TV.E.		236	
Le-3	181	Q.F--	E.R..E.....	HA.VT..NGD	AA.T.VVHQL..	NI.GF		238	
Le-4		SCR.....Q.F--	D.R..E.....	HS.VT..DGD	AA...WAHQL..	NII.F			
		metalloprotease							
VLAIP-A	241	YRVLNIIY	IALGLEIWNNGDKIN	VLPETKVTL	DLFGKWRERDL	LNRRKHDNAQL	LT	DINF	300
VLAIP-B	240	..F.....	VAV...SK..L..	TSSAYD...	S..E.....	KR.....	G...		299
VLFXA-HC	237	FIP...RLT	I.V.F.C.R.L...	TSSADD...	S..E..GS.....	KR.....	F..MK.		296
Le-3	239	..D..VH.T	SAV.V.T...L...	Q.AAS...N...	E.....	M..H....	G.DL		298
Le-4		..D..VH.T	SAV.V.T...L...	Q.AAS...N...	E.....	M..H....	A..L		
VLAIP-A	301	NGPTAGLGY	VGSMCDPQYSAGIV	QDHNKVNFL	VALAMAH	EMGHNLGMEHDEI	HCTCGAKS		360
VLAIP-B	300	...S..R.F..	R..Q.K..V.....	S.IYL...S.....	D..R.D.....				359
VLFXA-HC	297	DLS.L.IT	FLDG..QAYR.V...	E.GNK..KT.VI...	L.....Y..	RKN.I.NDS.			356
Le-3	299	DDNII..A	DD....R..V.....	SAIIR...VT...	L.....N..	GDQ.N...NG			358
Le-4		DDNTI..A	NEG....K..V.....	SAI.RM..AT....	-.....D..	GNQ.N...NG			
VLAIP-A	361	CIMSGT	LSCEASIRFSNCSREEHQY	LINKMPQCILNKPLK	TDIVSPA	VCNGNYLVELGED			420
VLAIP-B	360	I.R..	T.YL..D.....	R.....		F..V..E			419
VLFXA-HC	357	...AV..	SQP.KL...NH	DYRR..TTYK.K...	P..RK..A..PI...	EIW.E..E			416
Le-3	359	.V..VV	IEQR.YQ..D..	KNKY.T..T.RN.....	Q..R..T..TP.S..E	LQ----			414
Le-4		.V..AVIT	QQR.YQ..D..	KNKY.T..T.HN.....	Q.				
		spacer							
VLAIP-A	421	CDCGSP	RDCQNPCNAATCKLTPGS	QCADGECCDQCKF	RAGTVCRPANG	ECDSV	DLCTG		480
VLAIP-B	420	AN..	DR..D.....	R..A..G..V..Y.....					479
VLFXA-HC	417	K.....	D.....	AE.GN.L..EK..IKT.....	R.RD...PEH...				476
Le-3	415	-----NSG...	DPV..QPRR.EH	VS.K..RN...L.....	KR.V.D-.MD.Y...				466
		Disintegrin-like							
VLAIP-A	481	QSAECP	TDQFQRNGQPCQNNNGY	CYSGTC	PIMGKQCISLFG	SATVAQ	DA	CFQFNSLGNE	540
VLAIP-B	480	K....	N.....E.....		S.....RR..H				539
VLFXA-HC	477	A.G	HA.....N.D...T.....	SR...E.S...E	QK.SY				536
Le-3	467	I.SD..	RNPYK						477
		cysteine-rich							
VLAIP-A	541	YGYCR	KENGRKIPCAQDV	KCGRLYCFDNL	PEHKNPCQIYYT	PSDENKGM	VD	PGTKCGDG	600
VLAIP-B	540	NT..	A...E.....L..SSG.....	I.....					599
VLFXA-HC	537	I.....	L..S.GN....	KMH.RCR.QH....	E.....E..				596
VLAIP-A	601	KACSSNR	QCVDVNTAY						616
VLAIP-B	600	MV.-..GK...	TI..						614
VLFXA-HC	597	.V.NNK.....							612

Table 1. Cysteiny residues in domains of *Vipera lebetina* venom metalloproteases (VLMP)

VLMP	Number of Cys residues in domains						
	signal	prepro	protease	spacer	disintegrin-like	cysteine-rich	lectin
<i>P-I</i>							
Le-4	NR	NR	6	NA	NA	NA	NA
<i>P-II</i>							
Le-3*	1	3	6	0	10	NA	NA
<i>P-III</i>							
VLAIP							
A-chain	0	3	7	1	14	13	NA
B-chain	1	3	7	1	14	13	NA
<i>P-IV</i>							
VLFXA							
HC	0	3	7	1	14	14	NA
LC1	NA	0	NA	NA	NA	NA	7
LC2	NA	0	NA	NA	NA	NA	8

NR = Not reported; NA = not applicable; HC = heavy chain; LC = light chain.

* Le-3 disintegrin-like part is separated from fibrinolytic protease in the crude venom.

A-chain (100% identical with Le-3 disintegrin-like part) from *V. lebetina obtusa* venom [18].

We cloned the cDNA (accession No. AY987816) encoding a lebetase isoform designated as Le-4. The cDNA was cloned from the *V. lebetina* venom gland cDNA library by colony hybridization as described by Siigur et al. [17]. In comparison with lebetase Le-3 construct, Le-4 lacks the disintegrin-like domain coding region (fig. 1). The translated sequence is 87% identical to Le-3 [17] and 83% identical to fibrinolytic enzyme from Tunesian *V. lebetina* venom [19]. Le-3 and Le-4 have high sequence identities with several other P-I and P-II class reprotolysins and moderate sequence identity (about 40–50%) also with P-III proteases. Both lebetase isoforms contain six conserved cysteines that form three disulfide bonds (table 1).

We have shown that N-termini of the two lebetase isoforms are blocked with pyroglutamate [16]. The blocked N-terminus is characteristic of many short snake venom metalloproteases [20]. The commercial *V. lebetina* snake venom batches differ in the content and character of isoforms of fibrinolytic enzyme. The cleavage sites of fibrinogen by lebetase isoforms are identical, but the level of proteolytic activity varies. Le-3 and Le-4 translated pro-

tein forms deduced from the corresponding cDNAs have the same predicted theoretical pI values as measured in the single venom. The content of isoforms in different venoms is rather variable. We cannot rule out that isoforms have some specific role in envenomation process and different functional activities.

A Heterodimeric Endothelial Cell Apoptosis Inducing P-III Type Protease

Levantine viper venom contains several P-III type proteases: homo- and heterodimeric enzymes. One of them, human endothelial cell apoptosis-inducing protease-VLAIP (*V. lebetina* apoptosis-inducing protease), was purified, cloned and characterized [21]. This metalloprotease is a heterodimeric glycoprotein with molecular mass of about 106 kDa. The protease hydrolyzes azocasein, fibrinogen, and oxidized insulin B-chain. In contrast to lebetase, VLAIP cleaved only Ala¹⁴-Leu¹⁵ bond in oxidized insulin B-chain. Bradykinin was cleaved at position Pro⁴-Met⁵ by both proteases. VLAIP readily hydrolyzes the A α -chain and more slowly the B β -chain of fibrinogen. VLAIP does not cleave fibrin.

Internal sequences of VLAIP were determined using LC MS/MS technique, and oligonucleotides were designed and used as primers for cDNA cloning from *V. lebetina* venom gland cDNA library. The complete amino acid sequences of the two different monomers of VLAIP were deduced from the nucleotide sequences of cDNAs encoding these proteins. The full-length cDNA sequences of the VLAIP-A and VLAIP-B encode open reading frames of 616 and 614 amino acids respectively that include signal peptide, propeptide and mature metalloproteinase with disintegrin-like and cysteine-rich domains (fig. 1). Both VLAIP precursors have the 'cysteine switch' motif (PKMCGV) in the prodomain. This motif is involved in the activation of snake venom metalloproteases and matrix metalloproteases. Direct N-terminal sequencing of VLAIP resulted in no sequence, suggesting that the amino terminus of the protein is blocked. Many snake venom metalloproteases including lebetase have been reported to contain pyroglutamate as the amino terminal amino acid [15]. Proceeding from the conserved intrachain disulfide bond pattern of 6-Cys metalloproteases, the S-S bridges in VLAIP chains should look as follows: Cys³¹⁴-Cys³⁹⁴, Cys³⁵⁴-Cys³⁷⁸ and Cys³⁵⁶-Cys³⁶¹. Cys³⁶⁹ remains free and could be used for formation of interchain disulfide bond between the two monomers. Cys³⁶⁹ seems to be typical for vascular apoptosis-inducing

proteases, as other snake venom metalloproteases have different amino acids in this position. The disintegrin-like domain contains a ECD motif that is characteristic for many P-III type reprotolysins [22, 23] and also for ADAM metalloproteases [24]. The regions of ECD motif are identical for both VLAIPs (fig. 1), this region might be essential for binding to integrins. There is one potential N-glycosylation site in VLAIP-A and four in VLAIP-B in the deduced sequence. VLAIP belongs to the metalloprotease/disintegrin family of reprotolysins and has high identity with the proteins that induce apoptosis of endothelial cells [25, 26].

Treatment of HUVEC cells with VLAIP induces changes in the attachment of cells to the substrate and causes cell death. VLAIP inhibits endothelial cell adhesion to extracellular matrix proteins fibrinogen, fibronectin, vitronectin, collagen I, and collagen IV. The induction of apoptosis by VLAIP was shown by means of a typical DNA fragmentation pattern of apoptotic cells. The effects of VLAIP are cell type specific since VLAIP revealed only slight changes in the attachment of HeLa and NIH3T3 cells to the substrate [21]. VLAIP from *V. lebetina* snake venom is a potent promoter of apoptosis in vascular endothelial cells. VLAIP might be used as a tool for studying the functional mechanism of vascular endothelial cells.

VLFXA Is the Representative of P-IV Class Protease

V. lebetina venom contains specific coagulant Factor X activator (VLFXA) that cleaves the Arg⁵²-Ile⁵³ bond in the heavy chain of human factor X [27]. VLFXA is a glycoprotein that is composed of a heavy chain and two light chains linked by disulfide bonds. The complete amino acid sequences of the three chains of the factor X activator from *V. lebetina* snake venom are deduced from the nucleotide sequences of cDNAs encoding these chains [11]. The full-length cDNA sequence of the HC encodes 612 amino acids that include signal peptide, propeptide and mature metalloproteinase with disintegrin-like and cysteine-rich domains (fig. 1). The light chain LC1 of VLFXA contains 123 amino acids, the light chain LC2 135 amino acid residues. Both light chains belong to the class of C-type lectin-like proteins. The structural identity of the native VLFXA and the sequence translated from cDNA was demonstrated on the basis of the N-terminal analysis, electrospray tandem mass spectrometry and MALDI-TOF MS analysis of tryptic fragments of

purified VLFXA. The cDNA cloning of VLFXA clearly demonstrates that heavy and light chains are synthesized from different genes. The structure of VLFXA closely resembles that of the factor X activating enzyme from *V. russellii* venom (RVV-X) [28] having three polypeptide chains bound by S-S-bridges. While LC2 contains 8 cysteines and LC1 7 cysteines, LC1 might be connected with the heavy chain via LC2 (HC-LC2-LC1). The catalytic mechanism of VLFXA may be the same as proposed by Morita for RVV-X [29]. The heavy chain should have metalloprotease activity since it contains three domains characteristic to PIII-type metalloproteases. LC1 and LC2 resemble heterodimeric C-type lectin-like proteins and are very similar to IX/X binding protein [30]. The regulatory subunit (LC1+LC2) binds to the Gla-domain of factor X and then the catalytic subunit (HC) cleaves the Arg⁵²-Ile⁵³ bond in the heavy chain of factor X releasing the active factor Xa. Light chains serve as regulator regions that determine the specificity of binding of VLFXA to factor X.

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