THESIS ON NATURAL AND EXACT SCIENCES B63

# Purification, Characterization and Specificity Studies of Metalloproteinases from *Vipera lebetina* Snake Venom

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*Declaration*:

Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any degree or examination.

Signature of candidate:

Date:



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**ORIGINAL PUBLICATIONS**

This thesis is based on the following publications, which will be referred to in the text by their Roman numbers.

**I** **Trummal, K**., Vija H., Subbi, J., Siigur, J. (2000) MALDI-TOF mass spectrometry analysis of substrate specificity of lebetase, a direct-acting fibrinolytic metalloproteinase from *Vipera lebetina* snake venom. Biochim. Biophys. Acta 1476, 331-336.

**II** Siigur E., Tõnismägi K., **Trummal, K.**, Samel M., Vija H., Subbi J., Siigur J. (2001) Factor X activator from *Vipera lebetina* snake venom, molecular characterization and substrate specificity. Biochim. Biophys. Acta 1568, 90-98.

**III** Siigur J., Aaspõllu A., Tõnismägi K., **Trummal, K.**, Samel M., Vija H., Subbi J., Siigur E. (2001) Proteases from *Vipera lebetina* venom affecting coagulation and fibrinolysis. Haemostasis 31, 123-132.

**IV** Siigur J., **Trummal, K.**, Tõnismägi K., Samel M., Siigur E., Vija H., Tammiste I., Subbi J. (2002) Use of MALDI-TOF mass spectrometry for specificity studies of biomedically important proteases. Spectroscopy 16, 161-170.

**V** Siigur E., Aaspõllu A., **Trummal, K.**, Tõnismägi K., Tammiste I., Kalkkinen N., Siigur J. (2004) Factor X activator from *Vipera lebetina* venom is synthesized from different genes. Biochim. Biophys. Acta 1702, 41-51.

**VI** **Trummal, K**., Tõnismägi K., Siigur E., Aaspõllu A., Lopp A., Sillat T., Saat R., Kasak L., Tammiste I., Kogerman P., Kalkkinen N., Siigur J. (2005) A novel metalloprotease from *Vipera lebetina* venom induces human endothelial cell apoptosis. Toxicon 46, 46-51.

**VII** Siigur J, Tõnismägi K, **Trummal K**, Aaspõllu A, Samel M, Vija H, Subbi J, Kalkkinen N, Siigur E. *Vipera lebetina* venom contains all types of snake venom metalloproteases. Pathophysiol Haemost Thromb. 2005; 34:209-214.

## INTRODUCTION

Nature has been a source of medicinal products for thousands of years, among which snake venoms form a rich source of bioactive molecules, such as peptides, proteins and enzymes with important pharmacological activities.

All the known advanced snake species are venomous. Most of these snakes are found in the superfamily *Colubroidea* that also includes the families Elapidae (incl. hydrophidae, cobras, kraits, coral snakes, sea snakes) and Viperidae (vipers and pit vipers). Their venoms are a wide mixture of proteins and peptides (90–95%), also including amino acids, nucleotides, free lipids, carbohydrates and metallic elements bound to proteins (5%) (Heise et al 1995, Fry et al 2004, Russell et al 1980, Tu et al 1988). Studies employing two-dimensional SDS-PAGE on viperid venoms have demonstrated that a typical viperid venom may contain 200 up to perhaps 1000 proteins (Fox et al 2002, 2006).

Snake venom protein constituents may present different biological activities that affect physiological processes such as neurotransmission, the complement system and homeostasis (Gold et al 2002, Stocker 1990, Mion et al 2002). These venoms can act in more than one system at the same time and they may present antigenic effects (Mion et al 2002, Lewis and Gutmann 2004, Gornitskaja et al 2003). Viperidae family venom molecules are good examples, such as in haemeostasis, where they act as pro- and anticoagulant factors, and also as inducers and inhibitors of platelet aggregation (Markland 1997, 1998a, Braud et al 2000, Lu et al 2005). Due to their diversity, the proteins from the Viperidae family members are classified in the following categories: serine proteinases, metalloproteinases, phospholipases $A_2$ ($PLA_2$), C-type lectins and disintegrins from which only the first three groups display enzymatic activity (Russell 1980, Markland 1997).

Among the Viperidae serine proteinases are (i) thrombin-like enzymes that convert fibrinogen into fibrin (Markland 1998a, Nahas et al 1979), such as batroxobin in *Bothrops atrox* (Castro et al 2004), crotalase and gyroxin in *Crotalus durissus terrificus* (Nahas et al 1979, Stocker and Barlow 1976), and LMTL in *Lachesis muta* venoms (Raw et al 1986); (ii) factor X or factor V activators such as those described in *Bothrops jararaca* and *B. atrox* venoms (Magalhães et al 1981, Rosing et al 2001); (iii) prothrombin activators present in bothropic venoms (Tans and Rosing 2001); and (iv) platelet activators such as that found in *B. atrox* and *B. jararaca* venoms (Kini et al 2001, Niewiarowski 1977).

Snake venom metalloproteinases are zinc-dependent enzymes that induce hemorrhaging by directly affecting capillary blood vessels and their interaction with endothelial cells. They cleave basement membranes, leading to blood extravasation that occurs through gaps formed in endothelial cells (Serrano et al 1995). Therefore, these metalloproteinases such as BaH1 and BaP1 from the venom of the snake *Bothrops asper* present a hemorrhagic effect (Kamiguti et al 1998). This ability also induces myonecrosis and plays a vital role in the significant local inflammatory response of the envenomation (Tans and Rosing 2001, Serrano et al 1995, Rucavado et al 1995).

Snake venoms also contain several peptides. They may vary from presenting neurotoxic (Mion et al 2002, Francischetti et al 1997, Harvey et al 2001), cardiotoxic (Tsetlin et al 2004) or even an inhibitory platelet profile (Fry et al 2004, Russell 1980, Gold et al 2002, Ducancel 2002). In the group of peptides with inhibitory platelet activity, the disintegrins, also known as RGD peptides (molecules containing the Arg–Gly–Asp sequence), are integrin antagonists. They act as potent inhibitors of platelet aggregation by binding specifically to integrins present on cell membranes of not only

platelets (Weis et al 1998, Kini 1989) but also metastatic cells (Ducancel 2002, McLane et al 1998).

Finally, it is possible to observe the presence of other protein compounds in snake venoms. Those include cysteine-rich secretory proteins (CRISPs), which inhibit smooth muscle contraction and cyclic nucleotide-gated ion channels (review Yamazaki and Morita 2004). There are also phosphomonoesterases, phosphodiesterases, arginine esterases, hyaluronidases, L-amino acid oxidases, 5′- and NAD-nucleotidases and acetylcholinesterases in snake venoms (reviews Fry et al 2004, Russell 1980, Matsui et al 2000). Interestingly, the concentration and distribution of all snake venom proteins and peptides vary from individual to individual, species to species, genus to genus and family to family, probably due to their features, feeding and environmental conditions (Braud et al 2000, Du and Clemetson 2002, Tsai et al 2003).

At the end of the last millennium, the development of therapeutic drugs made a significant improvement to the understanding of the mechanisms of action and structure–function relationship of important biological molecules (Clemetson et al 2001, Monteiro et al 1998). Snake venoms, having a broad spectrum of biochemical, toxicological, physiological and pharmacological activities, are of biological interest as a potential source of active compounds that could act as (or be used as a prototype for) (i) therapeutic agents (Pal et al 2001); (ii) research tools for use in the diagnosis of several diseases (Pal et al 2001, Bailey and Wilce 2001, Marsh 2001); and/or (iii) in basic research for understanding physiological and pathological processes (Marsh 2001, Andrews et al 2001, Wisner et al 2001).

One of the most successful examples of using snake venom as a source for searching for drug prototypes also involved venom from the Viperidae family. The first venom-based anti-hypertensive drug captopril discovered in 1975 from the venom of Brazilian viper (*Bothrops jararaca*) also formed the first oral angiotensin-converting enzyme (ACE) inhibitor. Captopril was a breakthrough not only in management of blood pressure, but also as an early example of structure-based drug design, a revolutionary new approach to pharmacology (Patlak 2004).

Clearly snake venom peptides have the potential for practical and therapeutic use. However, enzymes and proteins are also very important as some of them are described as laboratory diagnosis reagents. Factor X and factor V activators from Russell's viper venom (RVV-X and RVV-V) and ecarin from *Echis carinatus* venom are proteins used for factors X and V, and prothrombin determination in blood, respectively (Magalhães et al 1981, Rosing et al 2001). Due to their characteristics, RVV enzymes have been used for the improvement of the detection of von Willebrand disease (Gold et al 2002, Nahas et al 1979). Similarly, snake venom thrombin-like enzymes (SVTLEs) are very useful for blood analysis of heparin-treated patients since they are not affected by heparin in the same way as thrombin, a key enzyme of the coagulation cascade (Markwardt 2001). SVTLEs and snake venom proteases presenting fibrinolytic activity acting on coagulation contributed to the study of the treatment of vascular thrombosis. Included in this group are batroxobin (Defibrinase R) from *B. atrox* venom and ancrod (Arvin R) from *Calloselasma rhodostoma* venom, currently used for controlled depletion of fibrinogen (Markwardt 2001, Bell 1997). They act as selective antithrombotic agents on deep vein thrombosis, peripheral arterial diseases and on vascular surgery (Bailey and Wilce 2001, Markwardt 2001, Bell 1979). In 2001, alfimeprase, produced as a truncated recombinant from fibrolase, was produced and introduced into clinical trials (Toombs 2001). It is presently in a phase II clinical trial for two indications: (a) treatment of peripheral arterial occlusions and (b) clearance of occluded vascular excess catheters, in direct competition with plasminogen activators

(Swenson et al 2004).

In the last decade, several snake venom compounds were used as important tools for the understanding of human physiological systems (Meier and Stocker 1991, Vogel et al 2004). Due to their similarity to physiological molecules, studies on myoblast fusion and fertilization, and matrix metalloproteinase (ADAMs)–cell interactions have been performed using the homologous snake venom metalloproteinases and peptide neurotoxins in order to characterize human cancers and small lung carcinoma. These studies are good examples of the use of snake venom molecules in basic research (Kamiguti et al 1998, Rucavado et al 1995, Marsh 2001, Meier and Stocker 1991, Vogel et al 2004).

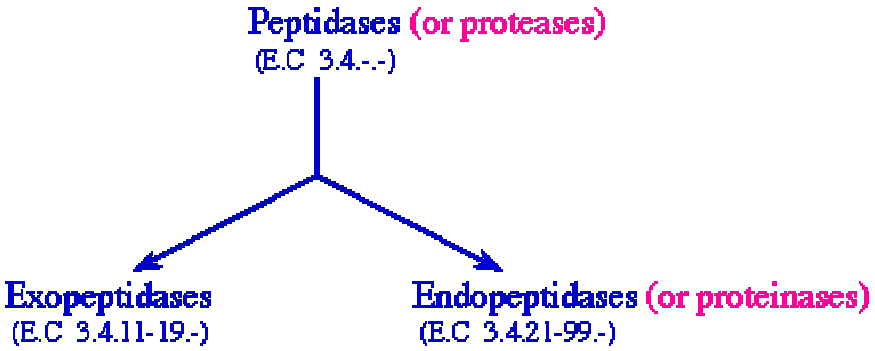
Due to SVMPs' similarity to several MMPs and ADAMs, their effects on adhesion and migration of different cell types have also been subject to intense investigation, particularly as potential therapeutic tools for cancer treatment, inhibiting metastasis and angiogenesis (Beviglia et al 1995, Cominetti et al 2003, Correa et al 2002, Schmitmeier et al 2003, Sheu et al 1997, Soszka et al 1991, Tomaczuk et al 2003).

In our laboratory several serine and metalloproteinases have been purified from *Vipera lebetina* venom. Three metalloproteinases that may have biomedical importance were chosen for further studies. Two of them - factor X activator (VLFXA) and endothelial cell apoptosis inducing proteinase (VLAIP) - have been purified during this work. Lebetase for specificity studies was isolated according to the method of Siigur and Siigur (1991).

## 1. REVIEW OF THE LITERATURE

### 1.1. Classification of proteinases

The International Union of Biochemistry and Molecular Biology (1984) has recommended to use the term *peptidase* for the subset of peptide bond hydrolases (subclass E.C 3.4.). The widely used term *protease* is synonymous with *peptidase*. *Peptidases* comprise two groups of enzymes: the endopeptidases and the exopeptidases, which cleave peptide bonds at points within the protein and remove amino acids sequentially from either N- or C-terminus respectively. The term *proteinase* is also used as a synonym word for *endopeptidase* and four mechanistic classes of proteinases are recognized by the IUBMB as detailed below. Thus, the modern scheme of nomenclature is:




Proteinases are classified according to their catalytic mechanisms. There are currently six classes of proteases:

- Serine proteinases
- Threonine proteinases
- Cysteine proteinases
- Aspartic acid proteinases (*e. g.* plasmepsin)
- Metalloproteinases
- Glutamic acid proteinases

The threonine and glutamic acid proteinases were not described until 1995 and 2004, respectively. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the character of a polar side-chain residue (serine, cysteine and threonine peptidases) or a water molecule (aspartic acid, metallo- and glutamic acid peptidases) nucleophilic so that it can attack the peptide carbonyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine or threonine as a nucleophile (Barrett et al 2003).

Proteases occur naturally in all organisms and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g., the blood clotting cascade, the complement system, apoptosis pathways, and the invertebrate prophenoloxidase activating cascade). Peptidases can break either specific peptide bonds (*limited proteolysis*), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (*unlimited proteolysis*). The activity can be a

destructive change abolishing a protein's function or digesting it to its principal components; it can be an activation of a function or it can be a signal in a signalling pathway.

The metalloproteinases may be one of the oldest classes of proteinases and are found in bacteria, fungi as well as in higher organisms. They differ widely in their sequences and their structures but the great majority of enzymes contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of the activity. Bacterial thermolysin has been well characterized and its crystallographic structure indicates that zinc is bound by two histidines and one glutamic acid. Many enzymes contain the sequence HEXXH, which provides two histidine ligands for the zinc whereas the third ligand is either a glutamic acid (thermolysin, neprilysin, alanyl aminopeptidase) or a histidine (astacin). Other families exhibit a distinct mode of binding of the Zn atom. The catalytic mechanism leads to the formation of a non covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group (Fersht 1999).

Snake venoms are a rich source of proteolytic enzymes which belong mainly to metalloproteinase and serine proteinase classes. Recently a new group of putative toxins, the renin-like aspartic proteases was reported (Wagstaff and Harrison 2006).

### 1.2. Snake venom metalloproteinases

**Reprolysins and ADAMs**

The snake venom metalloproteinases (SVMPs) are widely distributed in Viperidae and Crotalidae snakes and have been estimated to comprise on average 20-40% of the total venom proteins (Bazaa et al 2005). The snake venom metalloproteinases are members of the Reprolysin family of metalloproteinases. The term reprolysin was proposed by Bjarnason and Fox in 1994 to refer to a subfamily of the M12 family of metalloproteinases comprised of snake venom metalloproteinases (SVMPs) and ADAMs (*A D*isintegrin *A*nd *M*etalloproteinase) which minimally share homologous metalloproteinase domains, but in many instances also share homologous domains carboxy to the proteinase domain (Bjarnason and Fox 1994, 1995, Fox and Long 1998). The term reprolysin was meant at the time to connote the fact that some members of the subfamily belong to reptiles (*rep*) whereas others belong to a group of proteinases initially described in mammalian reproductive tissues (*repro lysin*) (Bjarnason and Fox 1994). However, it is now recognized that there are representatives of ADAMs proteins in a wide variety of organisms and tissues (White 2003). ADAMs are multidomain proteins that are minimally comprised of metalloproteinase and disintegrin-like domains. Most ADAMs possess additionally EGF-like, transmembrane and cytoplasmatic domains and therefore are primarily membrane associated, whereas SVMPs are secreted. ADAMs and SVMPs are members of the Metzincin family which also includes the ADAMTS proteins, the matrixins or matrix metalloproteases, astacins, serralysins, snapalysins and leishmanolysins. The name Metzincins is related to a conserved Met residue in a β-turn downstream the zinc binding motif. Unlike their snake venom relatives, ADAMs are expressed as transmembrane cell surface proteins. The domain structure of ADAMs suggests that these proteins possess both proteolytic and adhesive functions. Several members of the ADAM protein family have been shown to be involved in ectodomain shedding of many important cell surface proteins resulting in the release of biologically active soluble factors. The carboxyl-terminal

domains, especially the disintegrin-like domain of ADAMs, have been demonstrated to support cell adhesion. The disintegrin-like domains of many ADAMs are capable of acting as integrin ligands (Bridges and Bowditch 2005). The ADAM disintegrin-like domains are homologous to small non-enzymatic peptides – disintegrins - isolated from the venom of snakes that function as antagonists of integrins. Integrins are a widely distributed superfamily of noncovalent heterodimeric glycoproteins that play a vital role in cellular adhesion, migration, and signal transduction. Integrins known to interact with ADAM disintegrin-like domains include α4β1, α4β7, α5β1, α6β1, α9β1, αvβ3, and αvβ5. This integrin mediated interaction of the disintegrin-like domains with the cell surface suggests that ADAMs may function as cellular counter receptors (Bridges and Bowditch 2005).

Many of the ADAMs have functional proteinase domains; however, some ADAMs lack the characteristic HEXGHXXGXXHD catalytic site in their metalloproteinase domain and hence lack proteinase activity. ADAMs are best characterized as sheddases being capable of the proteolytic release of ectodomains of cell surface proteins to regulate physiological and developmental processes (Seals and Courtneidge 2003). ADAM-17 (TACE; TNF-α converting enzyme) is one of the best characterized due to its key function as the primary sheddase for tumor necrosis factor-α, as well as other surface proteins (Black et al 1997, Blobel 2000). ADAMs are also known to cleave and remodel extracellular matrix and/or growth factors bound to matrix giving rise to products which can themselves modulate biological processes such as cell migration and growth (Alfandari et al 2001). As more ADAMs are discovered (currently 40 ADAMs are known) and are better characterized as to their biological activities it is likely that this class of metalloproteinases will be viewed as a multifunctional, somewhat redundant group of proteinases intimately involved in a large variety of cellular and developmental process.

The ADAMTSs (*A D*isintegrin *A*nd *M*etalloproteinase with *T*hrombospondin *M*otifs) are similar in respect to the ADAMs in that they are also diverse and widespread (Kuno et al 1997). There are 13 ADAMTS genes known and a further subgroup with three members termed the ADAMTSL (ADAMTS-like) (Porter et al 2005). Members of the ADAMTS group have biological activities involved in matrix protein processing and degradation, proteoglycan degradation, inhibition of angiogenesis, blood coagulation, homeostasis, fertility and inflammation (Porter et al 2005).

### 1.2.1. Structure based classification and multiple domain organization

The SVMPs comprise the other subgroup of the reprolysins. It was established that some of these venom proteinases were inhibited by EDTA (Satake et al 1963) and hence indicated the presence of metalloproteinases in snake venoms. The SVMPs are the key toxins involved in venom-induced pathogenesis. SVMPs are synthesized in the venom gland as large multidomain proteins (Hite et al 1994) and are classified according to their multidomain organization in classes P-I to P-IV (Bjarnason and Fox 1994, 1995). The structural differences between these classes are the result of additional carboxy-terminal domains following the metalloproteinase domain.

#### 1.2.1.1. P-I class of SVMPs

Class P-I enzymes are synthesized in vivo as multimodular proteins that comprise a signal peptide, a prodomain and a metalloprotease domain. *The signal peptide* is

composed of 18 amino acids, mostly hydrophobic residues and functionally acts as a protein secretion marker. *The pro-domain* modulates the enzymatic activity through interactions with the catalytic domain. It is composed of about 200 amino acid residues and is highly conserved among the SVMP members. *The metalloprotease (MP) or catalytic domain* is composed of 200-215 amino acids, has metal-dependent endopeptidase activity and is less conserved than the pro-domain. For the P-Is, the metalloproteinases all have the zinc binding active site motif HEXXHXXGXXH as well and the Met-Turn sequence CI/VM and as such all likely function as active proteinases.

### 1.2.1.2. P-II class of SVMPs

P-II class SVMPs have additionally C-terminal *disintegrin domain*. Most disintegrins are released from P-II SVMP precursors and have an RGD-motif that is relevant for β3 (e.g. αIIbβ3 platelet fibrinogen receptor, and αVβ3, vitronectin receptor) and β1 integrin (e.g. α5β1, fibronectin receptor) binding (Rahman et al 2000, Okuda and Morita 2001, Yahalom et al 2002).

*The spacer peptide* was first defined as interdomain segment with 13-15 residues in length, between the metalloprotease and disintegrin domain. Based on the analysis of the latest results of the SVMPs' structures Fox and Serrano (2005) proposed a slightly modified classification scheme of the SVMPs (Fig. 1).

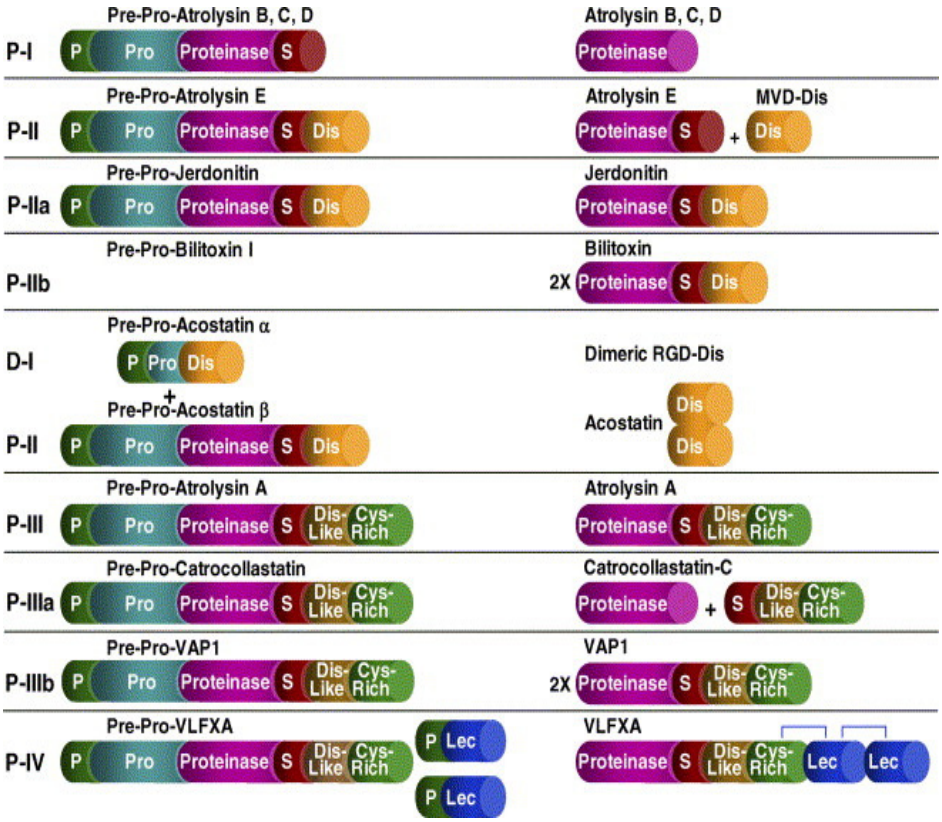



Fig. 1. Scheme of SVMP classes (reprinted from Toxicon 45, Fox JW, Serrano SMT. Structural considerations of the snake venom metalloproteinases, key members of the

M12 reprolysin family of metalloproteinases., p. 971, copyright (2005) by permission from Elsevier).

In this scheme there are subclasses within the P-II and P-III classes that reflect the potential for proteolytic processing as well as the formation of dimeric structures. The new classification contains additionally: P-IIa-P-II precursors whose mature forms do not release the disintegrin domain; P-IIb-P-II precursors whose mature forms comprise dimeric SVMPs; D-I precursors that do not contain metalloproteinase domain. Although the presence in venoms of disintegrins, the RGD containing peptides that block platelet aggregation, was known as early as 1987 (Huang et al 1987) their molecular source was unclear until cDNA sequences of SVMPs began to be solved (Hite et al 1992). From the cDNA analyses of these structures it became apparent that the disintegrins commonly found in Viperid venoms generally result from proteolytic processing of a precursor structure whereby the disintegrin 'domain' of the precursor is released giving rise to the active disintegrin (Shimokawa et al 1996). Only a few P-II SVMPs have been detected which in fact have not undergone proteolytic processing to release the carboxy disintegrin domain, including the dimeric bilitoxin-1 subclass P-IIb (Nikai et al 2000), and jerdonitin, subclass P-IIa (Chen et al 2003).

Fox and Serrano (2005) inspected the sequences of P-II members with particular attention to the position and number of cysteinyl residues that allowed for the further organization of the P-II class into several subclasses. One subclass is comprised by the three toxins that appear not to have their disintegrin domains processed from the metalloproteinase domain (bilitoxin-1, agkistin and jerdonitin). In the primary structure of these toxins there are characteristic cysteinyl residues found at positions 222 and 241, which are not found in these positions in the other P-II SVMPs. (The numeration of amino acid residues used in text are adapted from the review of Fox and Serrano, 2005). This suggests that the presence of cysteinyls 222 and 241 plays a role in arresting the proteolytic processing of the disintegrin from the metalloproteinase domain to maintain a two domain structure comprised of the metalloproteinase and disintegrin domains. Bilitoxin-1 as isolated from the venom of *Agkistrodon bilineatus*, has a native molecular weight of approximately 80 kDa (Nikai et al 2000).

The second group is represented by contortrostatin, acostatin-B and lebetase. Proteinases in this subclass are characterized by lacking cysteinyl residues at positions 233 and 235. Comparing protein structures of SVMPs isolated from venom having only a metalloproteinase domain with cDNA structures of P-II precursors to date only two P-II metalloproteinase lacking its disintegrin have been identified: lebetase from *Vipera lebetina* (Siigur and Siigur 1991) and HR2a from *T. flavoviridis* (Miyata et al 1989). Examination of the cysteinyl residues by Fox and Serrano (2005) in lebetase (Siigur et al 1996), contortrostatin and acostatin-B shows that all of these proteins lack cysteinyl residues at positions 233 and 235. In the case of both contortrostatin and acostatin-B, dimeric disintegrins have been isolated from the respective venoms. This would lead to the suggestion that given identities in cysteinyl positions of lebetase, contortrostatin and acostatin-B, there may be stable P-II metalloproteinase domains for the latter two in their respective venoms and in the case of lebetase, the cognate dimeric disintegrin may be present in its venom and in all cases these proteins have as yet to be isolated and characterized (Fox and Serrano 2005). Recently in our laboratory heterodimeric disintregrin was isolated from *Vipera lebetina* venom, one monomer of which is synthesized with lebetase (unpublished data).

A third group is represented by trigramin, flavoridin, TJM-1 and HR2a. This subclass is characterized by having a cysteinyl residue at position 168, but lacking the cysteinyl at position 164.

Recently there have been reported several cDNA structures that encode for a disintegrin alone (Okuda et al 2002, Francischetti et al 2004, Sanz et al 2006).

### 1.2.1.3. P-III class of SVMPs

The P-III class of SVMPs is perhaps the most interesting, at least in terms of the diversity of biological activities and structural features associated with this class (Fig.1). All P-III proteins have metalloproteinase domain, disintegrin-like and cysteine-rich domains. Until recently no RGD (Arg-Gly-Asp) motif was found in disintegrin domains of P-III SVMPs. Instead, alternative sequences such as DCD (Asp-Cys-Asp) or ECD (Glu-Cys-Asp) were found in the so-called *disintegrin-like domains* (Paine et al 1992, Hite et al 1994, Zhou et al 1995, Selistre-de-Araujo et al 1997, Souza et al 2000). Just recently, for the first time, the primary structure of an RGD-P-III class snake venom hemorrhagic metalloprotease BjussuMP-I, a SVMP isolated from the *Bothrops jararacussu* venom, has been reported (Mazzi et al 2006, in press). Likewise a KCD disintegrin-like motif in a snake venom P-III metalloproteinase precursor named bothrojarinh4 was first reported (Cidade et al 2006). The replacement of the Glu (E) residue found in most P-III metalloproteinases by the basic amino acid Lys (K) just beside the cysteine residue of the integrin-interacting motif may cause significant differences in the selectivity and/or potency of interaction with integrins, or even new biological activity which will require further study (Cidade et al 2006). The disintegrin-like domains of these proteins are considered capable of binding to integrin receptors and thus could serve a double function; targeting the toxin to a specific cell surface integrin and blocking the binding of normal ligands to the intergrin. However, the exact role(s) that this particular domain plays in the context of P-III SVMPs' biological activity has yet to be completely resolved.

*The cysteine-rich domain*, like the disintegrin domain, has high density of cysteins. With around 112 residues in length, it is located C-terminally to the disintegrin domain in P-III SVMPs. Several functional activities have been ascribed to cysteine-rich domain. Jia et al. (2000) demonstrated using recombinant cysteine-rich domain from atrolysin A that the domain was a potent inhibitor of collagen-stimulated platelet aggregation, but not ADP-stimulated aggregation thereby indicating interaction with the $\alpha 2\beta 1$ integrin on platelets. Using synthetic peptides, Kamiguti et al (2003) were able to localize regions in the cysteine-rich domain that were involved in platelet aggregation inhibition. Furthermore, the cysteine-rich domain of atrolysin A has been shown to be important in collagen binding (Jia et al 2000). Thus, it seems that in the P-III SVMPs the cysteine-rich domain is likely to have a key role in the composite activity of the toxin and as is the case with the disintegrin-like domain, just what that role is in the overall function of the toxin will need to be dissected out from those of the other domains (Fox and Serrano 2005).

Most of the metalloproteinase domains in the P-III class have seven cysteinyl residues. They share conserved cysteinyl residues at positions 126, 166, 168, 173, 190 and 206. In a recent review, Fox and Serrano (2005) compared the primary structures of class P-III, and presumed that the seventh cysteinyl residue position in the metalloproteinase domain might be the major differentiating factor associated with their various activities. For examples, the processed P-IIIs jararhagin, bothropasin, acurhagin and catrocollastatin, which can undergo proteolysis/autolysis under

physiological conditions (Usami et al 1994, Shimokawa et al 1997 and Moura-da-Silva et al 2003), have the seventh cysteinyl residue at the position 195 in the metalloproteinase domain whereas in the case of the other P-IIIs (excluding kaouthiagin) their seventh cysteinyl residue is found at a variety of positions in the metalloproteinase domain. All four of these proteases (P-IIIa subclass in Fig. 1) can undergo proteolysis/autolysis during secretion or in the venom to produce a biologically active, two-domain product comprised of the disintegrin-like and cysteine-rich domains (DC domain). In the case of jararhagin and catrocollastatin there have been identified substantial quantities of the processed DC domains, jararhagin-C and catrocollastatin-C, present in the venoms, along with the unprocessed jararhagin and catrocollastatin as well (Usami et al 1994, Shimokawa et al 1997). The protease domains released from the DC domain apparently are unstable and have not been isolated intact. In another group of P-III SVMPs including HV1, VAP1, halysase and VLAIP, the corresponding cysteinyl residue was found at position 181, and all of these proteins showed potent vascular apoptosis-inducing activity (Masuda et al 2000, 2001, You et al 2003, Trummal et al 2005).

At least two P-III toxins are known to exist in the native state as dimers; VAP1 and HV1, subclass P-IIIb (Masuda et al 1998, 2001). Examination of their primary structures indicates that their 'seventh' cysteinyl residue is conserved at position 181; also noteworthy is that none of the other non-processed PIIIs has their seventh cysteinyl residues at this position. Thus, it is suggestive that the presence of the seventh cysteinyl residue at position 181 may be critical for the formation of dimeric PIII structures. Based on proofs mentioned above, Fox and Serrano (2005) divided these proteins into subclasses of P-IIIa and P-IIIb, respectively (Fig.1). The seventh cysteinyl residue of stejnihagin-A and stejnihagin-B from *Trimeresurus stejnegeri* venom, similar to HR1b, locates at position 103, unlike that of P-IIIa and P-IIIb SVMPs. The analysis suggested that stejnihagin-A, stejnihagin-B and HR1b might belong to another subclass of P-III SVMPs, named P-IIIc, though their characteristic activities need further investigation. (Wan et al 2006). Based on comparison of amino acid sequences similarity of deduced mature stejnihagin-A and stejnihagin-B with other SVMPs, a phylogenetic tree of class P-III and P-IV SVMPs was constructed (Wan et al 2006b). It revealed that these SVMPs were obviously classified into four groups. Group one contained jararhagin, bothropasin, acurhagin and catrocollastatin, which have been included as P-IIIa SVMPs. Group two contained HV1, VAP1, halysase, VLAIP-A and VLAIP-B that belonged to P-IIIb SVMPs. Stejnihagin-A, stejnihagin-B and HR1b formed a novel group, P-IIIc. The last group contained two activators of factor X, RVV-X and VLFXA (both are class P-IV SVMPs) (Takeya et al 1992, Siigur et al 2001) and a prothrombin activator ecarin (Nishida et al 1995), all of which possessed the seventh cysteinyl residue at position 69 in the metalloproteinase domain. The grouping derived from phylogenetic analysis is in accordance with the classification based on the seventh cysteinyl residue position mentioned above. (Wan et al 2006b).

#### 1.2.1.4. P-IV class of SVMPs

The P-IV class of SVMPs has only two representatives, RVV-X (Russelysin) and VLFXA (***Vipera Lebetina*** **F**actor-**X** **A**ctivator). This class is characterized as having the non-processed P-III structure (metalloproteinase, disintegrin-like, and cysteine-rich domains) and two *C-type lectin-like domains* in the quaternary structure connected to the main chain of the PIII by disulfide bonds (Fig. 1). The cDNA analysis of VLFXA (Siigur et al 2004) revealed that this protein is synthesized from three independent

RNAs that code for a P-III metalloprotease (heavy chain) and two C-type lectin-like proteins (light chains). Thus, for now it is more appropriate to believe that P-IV SVMPs are in fact a modification of P-III SVMPs. Thus, it would appear that the production of this P-IV is a post-translational event whereby the P-III-like protein disulfide bonds with the C-type lectin-like protein. The second C-type lectin-like protein is disulfide bonded to the C-type lectin-like protein disulfide bonded with the P-III-like structure.

Inspection of the sequences of RVV-X and VLFXA defines two sequence differences from the rest of the P-IIIs. RVV-X, VLFXA and ecarin all have their seventh metalloproteinase domain cysteinyl residue at position 69. In the cysteine-rich domain RVV-X, VLFXA (but not ecarin) have an additional cysteinyl residue at position 400. Thus, it would appear that the presence of the additional cysteinyl residue at position 400 is a key determinant in the classification of a sequence as belonging to the P-IV class. It is likely, but will require proof, that cysteinyl 400 is involved in disulfide bond pairing with a cysteinyl residue in one of the C-lectin-like structures (Fox and Serrano 2005).

In Table 1 is presented a summary of some of the biological activities that have been associated with some of the SVMPs representing the four structural classes. Most of the functional activities associated with the SVMPs are connected with the disruption of hemostasis; essentially pro- or anti-coagulatory. More recent studies have indicated other functionalities for the SVMPs including apoptotic and inflammatory. The hemorrhagic activity has been assigned to proteinases in the P-I, P-II and P-III classes. Thus, the presence or absence of certain domains does not necessarily denote hemorrhagic capability although in terms of potency, in general the P-III hemorrhagic SVMPs are the most potent of the three classes. Although a large collection of primary structures of SVMPs have been assembled, as yet, primary structural data in and of itself have proven insufficient in fully explaining the structural basis of hemorrhage (Fox and Serrano 2005).

Table 1. Biological activities of selected SVMPs

| **SVMP** | **Activity** | **References** |
|---|---|---|
| ***P-I*** | | |
| Atrolysin C | Hemorrhagic | Shannon et al 1989, Zhang et al 1994 |
| Acutolysin A | Hemorrhagic | Gong et al 1998, Liu et al 1999 |
| Bap1 | Hemorrhagic, myonecrotic; inflammatory | Gutierrez et al 1995, Rucavado et al 1995 |
| Fibrolase | Fibrinolytic | Markland 1996 |
| HT-2 | Hemorrhagic | Mori et al 1987, Takeya et al 1990 |
| Atroxase | Fibrinolytic | Willis and Tu 1988 |
| LHF-II | Hemorrhagic | Sanchez at al 1991 |
| H2-proteinase | Proteolytic, non-hemorrhagic | Takeya et al 1989 |
| HR2A | Hemorrhagic | Takahashi and Osaka 1970, Yamada et al 1999 |
| Graminelysin I | Apoptotic | Wu et al 2001 |
| Leucurolysin-a | Fibrinolytic | Bello et al 2006 |

| | | |
|---|---|---|
| Lebetase | Fibrinolytic | Siigur and Siigur 1991 |
| ***P-II*** | | |
| Atrolysin | Hemorrhagic | Hite et al 1992, Shimokawa et al 1996 |
| MT-d | Proteolytic | Jeon and Kim 1999 |
| Jerdonitin | Inhibition of platelet aggregation | Chen et al 2003 |
| Bilitoxin-I | Hemorrhagic | Imai et al 1989, Nikai et al 2000 |
| ***P-III*** | | |
| Atrolysin A | Hemorrhagic, inhibition of platelet aggregation | Fox and Bjarnason 1995, Jia et al 1997 |
| Catrocollastatin | Inhibition of platelet aggregation | Zhou et al 1995, 1996 |
| Jararhagin | Hemorrhagic, inhibition of platelet aggregation | Paine et al 1992, Kamiguti et al 1996 |
| HF3 | Hemorrhagic, activation of macrophage phagocytosis | Assakura et al 1986, Silva et al 2004 |
| HR1a | Hemorrhagic | Omori-Satoh and Sadahiro 1979, Kishimoto and Takahashi 2002 |
| HR1b | Hemorrhagic | Omori-Satoh and Sadahiro 1979, Kishimoto and Takahashi 2002 |
| Kaouthiagin | Cleavage of vWF, inhibition of platelet aggregation | Hamako et al 1998, Ito et al 2001 |
| VAP1 | Apoptotic | Masuda et al 1998, 2001 |
| HV1 | Apoptotic | Masuda et al 2001 |
| Acurhagin | Hemorrhagic, inhibition of platelet aggregation | Wang and Huang 2002 |
| Ecarin | Activation of prothrombin | Kornalik and Blomback 1975, Nishida et al 1995 |
| Berythractivase | Activation of prothrombin | Silva et al 2003 |
| VLAIP | Apoptotic | Trummal et al 2005 |
| Bjussu MP | Hemorrhagic | Mazzi et al 2006 |
| ***P-IV*** | | |
| RVV-X | Activation of factor X | Takeya et al 1992, Gowda et al 1994 |
| VLFXA | Activation of factor X | Siigur et al 2001, 2004 |

#### 1.2.1.5. Structure and function of the metalloprotease domain

At the present moment seven crystal structures from the P-I class of metalloprotease domains from snake venoms are available: adamalysin II from *Crotalus adamantus* (Gomis-Ruth et al 1993, 1994), atrolysin C from *Crotalus atrox* (Zhang et al 1994), H2 proteinase from *Trimeresurus flavoviridis* (Kumasaka et al 1996), acutolysin A from *Agkistrodon acutus* (Gong et al 1998), BaP1 from *Bothrops asper* (Watanabe et al 2003), TM-3 from *Trimeresurus mucrosquamatus* (Huang et al 2002) and non-hemorrhagic fibrinolytic metalloprotease complexed with tripeptide inhibitor from the

venom of *Agkistrodon acutus* (Lou et al 2005). The 3D structural superposition of these structures makes evident remarkable similarities and ellipsoidal shapes (Stöcker and Bode 1995). In general P-I crystal structures share several defining structural characteristics beginning with an overall shape of an oblate ellipsoid with a central five-stranded β-sheet mixed with α-helices along with the conserved Met-turn structure between the αD and αE helices. The structure is comprised by an upper and lower domain with the substrate-binding cleft running between them. The active site cleft divides the metalloprotease domain in a large N-terminal sub-domain and a smaller C-terminal sub-domain joined by two or three disulfide bonds. In fibrolase, a non-hemorrhagic SVMP from *Agkistrodon contortrix contortrix*, the disulfide bonds were determined as $C^{117}$-$C^{197}$, $C^{159}$-$C^{164}$ and $C^{157}$-$C^{181}$ (Manning 1995). The disulfide bonds in the C-terminus, as well as the packaging of the C-terminal helix are the stabilizing elements that ensure the globular structure.

Recently the first crystal structure of the P-III class metalloprotease has been revealed, namely for a homodimeric VAP1 metalloprotease (Takeda et al 2006). By solving crystal structures of VAP1, a snake venom homolog of mammalian ADAMs, the ADAMs' MDC architecture and a potential target-binding site is revealed.

Topologically, the SVMP catalytic domains are classified as α/β proteins with an open-sandwich appearance, whose tertiary structure is composed by a fixed succession of secondary elements including a five stranded β-sheet (where only strand 4 is anti-parallel) and a five α-helix (where the α-1 is the smallest with 4-6 residues and α-2 the longest with 16-21 residues (Gomis-Rüth 2003, Watanabe et al 2003). In the catalytic site the catalytic zinc is coordinated by 3 His residues ($His^{334}$, $His^{338}$ and $His^{344}$ and by a water molecule. The first two His residues lay on the α-4 helix while the last is in loop started by the $Gly^{341}$ residue. The main-chain angles of the Gly residue suggest that this residue is required for this position (Stöcker et al 1995). This loop also contains the Met-turn motif that might be relevant since mutations generate enzymes with decreased activity (Pieper et al 1997, Hege and Baumann 2001). The carbon ε (Cε) of the conserved Met is about 4-6 Å from hydrophobic atoms of the zinc-binding His residues and may contribute to the establishment of an environment for fixing these side chains, favoring the zinc binding. The uppermost of the active site cleft is formed by β4, the floor by the loop region where the Met-turn is located, and the lateral walls by α4. In few crystallographic structures the presence of a calcium ion can be observed at the opposite side of the active site. This can be relevant for stabilizing the globular packaging of the molecule keeping the N- and C-terminus closer (Bode et al 1994).

The resolution of metalloproteases complexes with substrate mimetic inhibitors shed light into the active site cleft sub-sites related to substrate docking. The sub-sites S3, S2, S1 (that bind substrate N-terminal residues regarding the scissile bond), and S1´, S2´ and S3´(that bind substrate C-terminal residues regarding the scissile bond) were defined and are useful for inferring enzymatic specificities (Bode et al 1994, Lovejoy et al 1994, Zhang et al., 1994, Gomis-Ruth et al 1998). General characteristics of these subsites are: (a) S3 is hydrophobic and formed by sheet β4 and β4-β5 loop; (b) S2 is a shallow depression in bottom of the cleft formed between the end of a α4 and the third zinc binding His; (c) the P1 substrate residues point their side chains outside the cleft; (d) S1´is a hydrophobic tunnel-like pocket related to substrate specificity. It is formed by the α4 helix and loop residues following the Met-turn (Watanabe et al 2003); (e) S2´and S3´ are formed mainly by α4 helix, the loop between β5 and α4 and the loop between the Met-turn and α5 helix. The features above described for the SVMP catalytic domain and substrate/substrate mimetic binding studies suggest that its

enzymatic activity is selective for extended substrates (Ramos and Selistre-de-Araujo 2006).

Some efforts have been made to correlate enzymatic kinetic constants with hemorrhagic activity, but here again additional data from more P-I toxins will be required to determine whether these correlations are in fact valid for all P-I toxins (Huang et al 2002). It would appear that attempts to correlate biological activities with tertiary structure have not been particularly fruitful; however, it is likely that as additional structures are solved, particularly of members from other classes, there may be discovered some underlying tertiary structural features that are important in defining particular biological activities.

### 1.2.2. Proteolytic specificity of SVMPs

Snake venoms contain a number of proteins - enzymes and peptides affecting hemostasis: nucleotidases, phosphodiesterases, L-amino acid oxidases, phospholipases $A_2$, metallo- and serine proteases, nonenzymatic C-type lectins and disintegrins (reviews Stocker 1990, Braud et al 2000, Lu et al 2005).

The disruption in the hemostatic system is the most studied biological effect of SVMPs. Regarding this aspect, SVMPs can be classified as hemorrhagic, coagulant and anticoagulant enzymes.

#### 1.2.2.1. Hemorrhagic SVMPs

Crotalidae and Viperidae venoms contain many metalloproteinases that degrade the blood vessel extracellular matrix (ECM) and thus affect hemostasis (reviews Gutiérrez et al 2005, Bjarnason and Fox 1995). The SVMP proteolytic activity is related to the induction of hemorrhage, inflammation and necrosis *in vivo* that characterize the local lesion usually observed in Viperidae snake envenomation (Laing et al 2003, Gutiérrez and Rucavado 2000). The hemorrhagic effect can be achieved by synergistic activities including the degradation of coagulation proteins, endothelial basal membrane and tissue ECM. Johnson and Ownby (1993) concluded that the hemorrhage could occur by two distinct mechanisms, *per rhexis* (loss of red blood cells through lysed or disrupted endothelial areas) or *per diapedesis* (loss of red blood cells through widened junctions of intact endothelial cells).

SVMPs exert their effects by degradation of proteins such as laminin, nidogen, fibronectin, collagen type IV and proteoglycans from the endothelial basal membrane (Baramova et al 1989, 1990, 1991, Shannon et al 1989). Blood coagulation proteins are also targets of their proteolytic activity, such as fibrinogen (Johnson and Ownby 1993, Selistre-de-Araujo et al 2000, Ramos and Selistre-de-Araujo 2004, Garcia et al 2004) and von Willebrand factor (Kamiguti et al 1996), enhancing the hemorrhagic state. Additionally, SVMPs can also inhibit the platelet aggregation (Laing and Moura-da-Silva 2005, Kamiguti 2005) and trigger the release of cytokines (Schattner et al 2005). These two effects associated with the proteolytic digestion of basal membrane are thought to be the major mechanism of SVMP-induced hemorrhage.

The Minimum Hemorrhagic Dose (MHD) assay was originally designed in 1960 (Kondo et al) to quantify the hemorrhagic potency of SVMPs in rabbits. MHD is defined as the minimum protein mass that is necessary to induce a hemorrhagic spot with 1 cm diameter, when applied intradermally, 2 h after injection. Despite the fact that most SVMPs have hemorrhagic activity (Johnson and Ownby 1993), some of them, when highly purified, are not hemorrhagic but conserve their proteolytic activity

(Ahmed et al 1990, Toombs 2001, Ramos et al 2003). A hypothesis for explaining these differences was proposed based on the specific cleavage of relevant peptide bonds of basal membrane proteins by hemorrhagic enzymes (Bjarnason and Fox 1994).

Many attempts were made aiming to predict the hemorrhage potency of SVMPs, including sequence alignment, phylogenetic reconstruction, comparison of predicted physical and chemical properties and motif scanning with no conclusive results (Paine et al 1992, Manning 1995, Takeya et al 1989, 1990, Gasmi et al 2000, Tsai et al 2000). Watanabe et al (2003) suggested that a loop region between the third zinc-binding His and Met-turn could be related to the hemorrhagic phenotype, due to the solvent exposure and the structural variability among SVMP members. The analyses of solved and modeled structures showed an indirect correlation between hemorrhagic potential and polar molecular surface area of P-I enzymes (Ramos and Selistre-de-Araujo 2004). In general terms, P-III SVMPs are more potent hemorrhagic toxins than SVMPs of other groups, particularly of class P-I, where a number of non-hemorrhagic proteinases have been characterized (see table 1). Even those P-I SVMPs that are hemorrhagic have reduced activity when compared with many P-III enzymes (Bjarnason and Fox 1994), the simple suggestion being that the additional domain structure characteristic of that class may be contributing to their greater hemorrhagic potencies. However, the detailed mechanism by which SVMPs disrupt microvessels remains elusive.

#### 1.2.2.2. Nonhemorrhagic fibrin(ogen)olytic metalloproteinases

Fibrinolytic and fibrinogenolytic activities have been described in venoms of Viperidae, Elapidae and Crotalidae snake families. Fibrinogenolytic enzymes can cleave the α-, β- and γ-chains of fibrinogen with variable chain specificity, thereby turning the protein unclottable (reviews Markland 1991, 1998a, b, Siigur and Siigur 1992, Swenson and Markland 2005). In some snake species the fibrinogenolytic activity is caused by proteolytic enzymes that have also hemorrhagic activity. In other venom species these two activities are caused by separate but closely related proteolytic enzymes. The most thoroughly characterized nonhemorrhagic fibrinolytic enzymes are fibrolase from *Agkistrodon contortrix contortrix* venom and lebetase from *Vipera lebetina* venom. Fibrinolytic snake venom enzymes are about 23 kDa metalloproteinases containing 1 mol of zinc per mol of protein. The tertiary structures of these fibrinolytic enzymes are stabilized by three disulphide bonds. Fibrolase and lebetase lyse fibrin by selective cleavage of peptide bonds in the α- and β-chains. The thrombolytic activity of fibrolase was successfully demonstrated in a canine model of reoccluding carotid arterial thrombosis (Markland et al 1994). Recent results show that recombinant form of truncated fibrolase, named alfimeprase has clinical potential for the treatment of heart attack.

Due to lebetase specificity studies in this work we characterize this enzyme in more detail.

##### 1.2.2.2.1. Lebetase

A fibrinolytic enzyme from the venom of *Vipera lebetina* (Levantine viper) has been purified by Siigur and Siigur (1991). This enzyme, named lebetase, is a metalloproteinase which readily cleaves α-chain and more slowly β-chain of fibrin and fibrinogen. The enzyme possesses direct-acting fibrinolytic activity and does not activate plasminogen. Antibodies against lebetase crossreact with different snake venom fibrinolytic components (Siigur et al 1996). The amino acid sequences of two

lebetase isoforms were deduced from lebetase coding cDNAs (Siigur et al 1996, Aaspõllu et al 2005). Lebetase is the first fibrinolytic enzyme ever sequenced from Viperidae venom gland. The lebetase isoforms belong to the P-I class proteins having a metalloproteinase domain only. The isoforms are synthesized from N-I class polynucleotides, comprising signal peptide, proenzyme and mature metalloproteinase domains in the translated sequence, or N-II class polynucleotides that additionally contain a disintegrin-like domain following the proteinase domain. In the latter case the enzyme is synthesized in venom gland as P-II class proteinase as based on the modified classification scheme of the snake venom metalloproteinases (Fox and Serrano 2005) and the posttranslational cleavage gives the mature P-I class protein. The sequence of the disintegrin-like part of lebetase clone Le-3 exists as a monomer in the heterodimeric disintegrin in the *V. lebetina* venom.

The primary structures of lebetase isoforms have extensive sequence homology with fibrolase and with several other small snake venom metalloproteinases (Siigur et al 1996, Aaspõllu et al 2005). Lebetase inhibited ADP- and collagen-induced platelet aggregation (Siigur et al 1998).

#### 1.2.2.3. Apoptosis inducing snake venom metalloproteinases

Apoptosis is a programmed physiological mode of cell suicide. The main characteristic morphological features (changes) of apoptosis include cellular shrinkage, membrane blebbing, nuclear condensation and DNA fragmentation, and disassembly of the cell into membrane-enclosed vesicles (apoptotic bodies). Apoptosis takes place during embryogenesis, during development of the nervous system and the immune system. Apoptosis is implicated in the homeostatic cell balance in normal adult tissues. Animals use apoptosis to eliminate extraneous or dangerous cells. Defects in the coupling of cell death and multiplication result in pathologies such as tumors or functional deficiencies (Golstein 1998). Studies on eukaryote (human) cells have shown that a number of related proteases (family of caspases) are involved in cell apoptosis. These proteases are usually synthesized as precursors that have little, if any, catalytic activity. The precursor is converted into an active enzyme by proteolytic processing. Activated caspases destroy particular intracellular proteins that protect living cells from apoptosis and also reorganize cellular structures indirectly by cleaving several proteins involved in cytoskeleton regulation, thereby killing the cell (Thornberry and Lazebnik 1998). Members of the caspase family play important roles as mediators in apoptosis. Some take part in the signalling pathways and others act as effectors in apoptosis (Nicholson and Thornberry 1997). They can be divided into initiator caspases and effector caspases by the presence and absence, respectively, of a large pro-domain in the amino-terminal region (Salvesen and Dixit, 1999). Among the initiator caspases, caspase-9 is involved in the apoptotic pathways arising from mitochondrial dysfunction (Green 2000). Caspase-8 and -10, which are also initiator caspases, participate in the pathways mediated by death receptors (Ashkenazi and Dixit 1998, Wang et al 1999). Those three initiator caspases play an important role in transducing specific apoptotic stimuli and transmitting apoptotic signals to effector caspases (Budihardjo et al 1999). *In vitro* studies have shown that caspase-9 can activate caspase-8 and -10 via caspase-3 (Slee et al 1999), and also that caspase-8 can activate caspase-9 and-10 (Alnemri et al 1996).

Endothelial cells play a critical role in vascular homeostasis such as blood coagulation, vascular contraction, and the control of vascular permeability. Human umbilical vein endothelial cells (HUVEC) express different integrins such as $\alpha_2\beta_1$, $\alpha_3\beta_1$,

$\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_v\beta_1$ on the surface of their membranes. The endothelial cell integrins interact with extracellular matrix (ECM) proteins. These interactions are important for vascular integrity, permeability and angiogenesis. Extracellular matrix proteins (e.g. fibronectin, vitronectin, fibrinogen etc.) as well as snake venom metalloprotease/disintegrin components contain the RGD (or other) sequence motif that recognizes integrin receptors involved in cell-matrix interactions (Luscinskas and Lawler 1994). There are many different factors known to affect endothelial cells' function, these factors include vasoconstrictors, vasodilators, anticoagulants, growth factors, cytokines, vascular endothelial growth factor (VEGF), TGF-β, TNF-α and components from snake venoms.

Snake venoms contain different proteins (L-amino acid oxidase, metalloproteases, disintegrins etc.) affecting apoptosis of normal (e.g. endothelial) and cancer cells (Ali et al 2000, Araki et al 1993, Suhr and Kim 1996, Torii et al 1997, 2000, Masuda et al 1998, 2000, 2001). Recently, several snake venom metalloproteinases have been reported to induce apoptosis of human endothelial cells: halysase from *Gloydius halys* venom (You et al 2003), agkistin from *Agkistrodon halys* (Wang et al 2003), HV1 from *T. flavoviridis* venom (Masuda et al 2001), VAP1 from *Crotalus atrox* venom (Masuda et al 1998 and Masuda et al 2000), and graminelysin I from *T. gramineus* venom (Wu et al 2001). A particular type of apoptosis named anoikis can be evoked by molecules that degrade the ECM as MMPs. Like MMPs, SVMPs degrade all major ECM proteins and are involved in shedding or blocking of integrin molecules which are largely related to cell/matrix interactions (Kamiguti et al 1996). Therefore, SVMPs can be useful tools for the study of cell physiology, particularly apoptosis and anoikis.

Both P-I and P-III SVMPs are able to induce apoptosis of endothelial cells. In the case of P-I enzymes, inhibition of catalytic activity abrogates their ability to induce apoptosis (Wu et al 2001). In contrast, halysase, a P-III enzyme from the venom of *G. halys* induces apoptosis even when catalytic activity is absent (You et al 2003).

VAP1, the vascular apoptosis-inducing protein 1 has been suggested to provoke some caspase-independent apoptotic cell death pathways for HUVECs (Maruyama 2005). Endothelial cell death due to deprivation of a survival factor is reported to be mediated entirely by caspases (Kondo et al 1996). Cell death induced by VAP1 may have a complicated mechanism. Several observations suggest that VAP1 induces apoptosis by activating some special signals, like as 'death receptor signals', other than cell detachment signals or stress signals. Thus the apoptosis induced by VAP1 may be considered a new category of endothelial cell apoptosis (Maruyama 2005).

The action of SVMPs on endothelial cells may involve at least two independent mechanisms: the catalysis of focal adhesion components leading to anoikis and/or a direct effect through receptor-mediated signal transduction initiated by the disintegrin domain. The resulting apoptotic effect would then reflect a balance between the action of catalytic and disintegrin activities

#### 1.2.2.4. Prothrombin activators

Snake venoms contain different prothrombin activators of metallo- and serine classes of proteases (reviews Rosing and Tans 1991, Hutton and Warrell 1993, Kini 2005). The activation of prothrombin to thrombin is accomplished *in vivo* by the prothrombinase complex consisting of serine proteinase factor Xa, a cofactor Va, assembled on negatively charged phospholipid membranes in the presence of $Ca^{2+}$ ions.

Snake venom prothrombin activators are classified into four groups based on their cofactor requirements. Group A and group B activators are metalloproteinases and they

are structurally unrelated to coagulation factors, group C and D prothrombin activators are serine proteinases (reviewed by Kini 2005). The best-known activator belonging to the group A is ecarin from *Echis carinatus* that requires no cofactors and efficiently converts prothrombin to meizothrombin (Kornalik and Blomback 1975, Nishida et al 1995). Group B prothrombin activators contain C-type lectin related subunit. Examples of group B activators are carinactivase-1, also isolated from *Echis carinatus* (Yamada et al 1996) and multactivase from *Echis multisquamatus* that also converts prothrombin to meizothrombin, but requires $Ca^{2+}$ ions for activity (Yamada and Morita 1997).

Until now, at least 13 prothrombin activating metalloproteases were discovered from snake venoms. These direct-acting prothrombin activators are generally single-chain PIII SVMPs (50-70 kDa) that cleave selectively the $Arg^{322}$-$Ile^{323}$ bond of prothrombin generating meizothrombin. The last is spontaneously converted to thrombin. Their activity is not influenced by non-enzymatic cofactors of the prothrombinase complex.

### 1.2.2.5. Factor X activators

Various factor X activating components are widely distributed in the venoms of Viperidae and Crotalidae. Factor X is activated by the venoms of a number of genera: *Daboia, Vipera, Cerastes, Echis, Calloselasma and Bothrops* (Yamada et al 1997). The venoms of *Daboia* snakes (*D. russelli russelli* and *D. russelli siamensis*) possess the highest level of factor X-activating activity. Factor X activators from different venoms have been characterized (reviews Morita 1998, Tans and Rosing 2001, Siigur and Siigur 2006). Viperidae and Crotalidae venom activators are mainly metalloproteases. Only two factor X activators are characterized from Elapidae venoms, both belonging to serine proteases. Most thoroughly investigated snake venom factor X activators are from *Vipera russelli* (now renamed *Daboia russelli*) - RVV-X, and *Vipera lebetina* (now renamed *Macrovipera lebetina*) - VLFXA. VLFXA is also the object of the present study and will be described in results and discussion.

These factor X activators have molecular sizes ranging from 38 to 102 kDa and are calcium-dependent. RVV-X (92.88 kDa), the first P-IV SVMP to be reported with such activity, is a glycoprotein with 13% of carbohydrate composed of one heavy chain (57.6 kDa) and two disulfide linked light chains (19.4 and 16.4 kDa). The activation mechanism of bovine FX by RVV-X does not differ from the physiological mechanism of activation (cleavage of $Arg^{51}$-$Ile^{52}$ bond) giving rise to the active serine proteinase FXaα. Conversion of FXaα to FXaβ is very slow in the case of low concentrations of RVV-X. The activation has an absolute $Ca^{2+}$ requirement. The effect of $Ca^{2+}$ on the activation is mediated by interaction of the cation with the substrate, rather than with the enzyme (Amphlett et al 1982). $Ca^{2+}$ initiates a conformational change in factor X that is a prerequisite for optimal proteolysis by RVV-X. This Ca-dependent conformational change requires the Gla domain (Skogen et al 1983). Factor X binds $Ca^{2+}$ by at least three so-called "tight" binding sites ($K_d$ $2.2x10^{-4}$ M) associated with the Gla residues, and 25 "weak" binding sites ($K_d$ $2.5x10^{-3}$ M) at other sites in the molecule (Esnouf 1978). Kosow (1976) showed that $Ca^{2+}$ influenced the $V_{max}$ for the activation reaction without any effect on the $K_m$. Lindhout et al (1978) demonstrated that des-γ-carboxy FX is activated by RVV-X only in the presence of $Ca^{2+}$ proving that "weak" Ca-binding sites also participate in the activation reaction (although the activation rate is 60 times lower). Skogen (1983) studied the activation of FX and Gla-domainless FX by RVV-X in the presence and absence of $Ca^{2+}$. Gla-domainless FX was a poor substrate for RVV-X though $K_m$ and $k_{cat}$ could be detected. RVV-X is not able to

activate factor X molecules bound to phospholipid bilayers (van Diejien et al 1981). In terms of its mechanism of action, RVV-X is the simplest activator of FX, requiring only $Ca^{2+}$ as a cofactor and having no feedback requirements (Jesty 1986).

## 2. MATERIALS AND METHODS

### 2.1. Materials

Bradykinin, kallidin (Lys-bradykinin), substance P, glucagon, neuraminidase were purchased from Serva (Heidelberg, Germany). 2,5-Dihydroxybenzoic acid (DHB), benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide (S-2222), fibrinogen and human factor X, CM-52 cellulose, fibronectin, vitronectin, collagen I, collagen IV, laminin, fibrinogen, insulin B-chain, streptomycin, heparin, MTT were from Sigma (St. Louis, MO, USA). The venom batches of *V. lebetina* were commercial preparations from Tashkent Integrated Zoo Plant (Uzbekistan). Sephadex G-100 (superfine), PBE-94, polybuffers, markers and ampholytes for isoelectric focusing were products of Pharmacia (Uppsala, Sweden). Bovine factor X and benzoyl-Ile-Glu-(Pip)-Gly-Arg-*p*-nitroanilide hydrochloride (S-2337) were purchased from Chromogenix (Mölndal, Sweden). Prothrombin was kindly provided by Dr. T. Ugarova. All peptides were synthesized at the Laboratory of Bioorganic Chemistry at the 100 μmol scale on an Applied Biosystems 431A Peptide Synthesizer using BOC (*t*-butyl-oxycarbonyl) chemistry as suggested by the manufacturer. FCS from PAA Laboratories (Austria), ECGS from Upstate (USA). DNA ladder marker was from Fermentas (Vilnius, Lithuania). Oligonucleotide primers were ordered from DNA Technology (Aarhus, Denmark). All other reagents used were of analytical grade.

### 2.2. Methods

#### 2.2.1. Peptide synthesis

Since its introduction in 1963 by Bruce Merrifield, solid-phase peptide synthesis (SPPS) has become the most successful method for synthesizing peptides. SPPS generally involves five steps: chain assembly, cleavage from resin and removal of side chain protecting groups, purification, additional chemical modification and characterization. All peptides were synthesized at the 100-μmol scale on Applied Biosystems 431A Peptide Synthesizer using BOC (*t*-butyl-oxycarbonyl) chemistry as suggested by the manufacturer. The *t*-BOC group that protects the α-amino group is removed at the beginning of every cycle by a weak acid, typically 50% TFA in DCM. After deprotection, the resin is washed with DCM to remove most of the TFA. Any remaining TFA and the protonated amino groups are neutralized with a dilute DIEA solution. The resin is then washed with NMP, the same solvent used in the coupling reaction. Before coupling, the carboxyl group of the amino acid must be activated. HOBt/DCC is used for standard activation of all amino acids on the Model 431A. During coupling, the activated *t*-BOC-amino acid reacts with the amino-terminal of the growing peptide chain. There are three coupling stages, defined by the solvent added at each step: 100% NMP, DMSO and DIEA. During the NMP stage, coupling reactions may exceed 99% completion, although in some cases coupling reaction completion may be much less. DMSO is added to enhance and improve solvation of the peptide-resin and increase coupling for difficult sequences. Enough DMSO is added to produce a solution with 15% DMSO and 85% NMP. During the last coupling stage, DIEA is added to further disrupt peptide-peptide hydrogen bonds and increase solvatation. The DIEA also neutralizes any protonated amine groups, which helps to accelerate the coupling reaction. A resin sample can be taken after coupling is finished. Once the resin sample is removed, the capping reaction begins. Capping uses a mixture of acetic

anhydride, DIEA and NMP to acetylate any unreacted amines and make them unavailable to react in future coupling cycles. This simplifies the purification process since it is easier to remove the shorter, capped peptides than the longer deletion peptides.

For cleavage of peptides from the resin support and removal of side chain protecting groups trifluoromethane sulfonic acid (TFMSA) was used. The peptide-resin was placed in a small (10-50 ml) round-bottom flask equipped with a micro stirbar. 150 µl of thioanisole: ethanedithiol (2:1) was added and the mixture was stirred for 10 minutes at room temperature. 1 ml of neat TFA was added and stirred for 5 to 10 minutes. 100 µl TFMSA was added dropwise with vigorous stirring and the mixture was allowed to react for 25 minutes at room temperature. When the reaction was completed cold diethyl ether was added to the flask to precipitate the peptide and the mixture was stirred for 1 minute. The content of the flask was transferred to a sintered glass funnel and the solid was washed with cold diethyl ether several times. A clean collection flask containing approximately 200 ml diethyl ether was placed under the funnel and the peptide on the frit was dissolved with a minimum volume of TFA, the peptide should go into solution easily. The peptide solution was filtered into the ether and the resin on the frit was washed twice with a minimum volume of TFA. The precipitated peptide/ether solution was cooled over ice. The precipitate was filtered through a sintered glass funnel (fine porosity) and washed with ether several times and lyophilized.

The purity of peptides was assessed by analytical reverse phase-high performance liquid chromatography (HPLC) and MALDI-TOF mass spectrometry. All peptide solutions were directly prepared in 0.05 M $NH_4HCO_3$, at concentrations of about 1–5 mg/ml, and kept frozen at −20 °C until use.

### 2.2.2. Monitoring of the enzymatic reaction

The enzymatic hydrolysis of peptides was carried out in 0.05 M $NH_4HCO_3$, pH 8.3, at 37 °C in Eppendorf tubes. In a typical experiment, 200 µl of 0.05 M $NH_4HCO_3$ solution of substrate (5 mg/ml) in an Eppendorf tube was thermally equilibrated to 37 °C in the thermostated rack. The reaction was started by addition of 15 µl of enzyme solution (1 mg/ml in 0.05 M $NH_4HCO_3$). At predetermined time intervals (0.5–24 h), the reaction mixture (50 µl) was diluted with 500 µl of $H_2O$ and 10 µl of 6 N HCl was added to stop the reaction. We applied a low enzyme–substrate ratio (1:3 – 1:70 on a weight basis) to determine the position of cleavage. A 0.5-µl amount of diluted mixture was used for MALDI-TOF mass spectrometry analysis.

### 2.2.3. MALDI-TOF mass spectrometry of peptides and their enzymatic cleavage products

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to analyze the mixtures of peptides obtained after metalloproteinase (lebetase, VLAIP or VLFXA) treatment. The MALDI mass spectra were measured with a home-built gridless time-of-flight MALDI mass spectrometer designed for maximum flexibility in use (National Institute of Chemical Physics and Biophysics). For this work, it was used in linear delayed extraction mode with 4 kV pulsed extraction and 24 kV total acceleration voltage. The 2-m flight tube was used. A double multichannel plate detector with conversion dynode was used for ion detection and Tectronics TDS 520 digitizing oscilloscope for data accumulation. An excimer

laser pumped dye laser working on 340 nm was used for desorption/ionization, and the delay between laser pulses was 130 ns.

The matrix used throughout this study was DHB. DHB (10 mg) was dissolved in 1 ml of a 1:1 mixture of 0.1% trifluoroacetic acid and acetonitrile for sample preparation. A 0.5 µl aliquot of this mixture was deposited on a stainless steel probe tip, mixed there with 0.5 µl of reaction mixture of peptides and enzyme, and allowed to dry at room temperature. External mass calibration was accomplished by using as peptide standards, substance P and bradykinin.

### 2.2.4. Purification of metalloproteases

#### 2.2.4.1. Gel filtration on Sephadex G-100 superfine

The crude venom of *V. lebetina* (1.5 g) was dissolved in 15 ml of 0.2 M ammonium acetate, pH 6.7. Insoluble material was removed by centrifugation (5000×*g* for 15 min) and the supernatant was applied to the column (2.8 × 128 cm) of Sephadex G-100 superfine equilibrated with 0.2 M ammonium acetate. The elution was carried out with the same solution at a flow rate 6.8 ml/h and fractions were collected at 1 h intervals.

#### 2.2.4.2. Ion exchange chromatography on CM-52 cellulose

The column (1.5×9 cm) was equilibrated with 0.2 M ammonium acetate, pH 5.5. Combined and concentrated by lyophilization or by ultrafiltration, fraction I–II from gel filtration (30 mg in 3 ml of 0.2 M ammonium acetate, pH 5.5) was applied to the column. Non-adsorbed material was washed with the equilibration solution. The VLAIP and factor X activating protein were eluted with a gradient of 0.2 – 0.5 M ammonium acetate, pH 5.5. The flow rate was 12 ml/h; fractions of 3 ml were collected.

#### 2.2.4.3. Ion exchange chromatography on TSK-DEAE HPLC

Ion exchange chromatography on TSK-DEAE HPLC was performed on a DuPont HPLC system with a variable wavelength detector at 280 nm. VLFXA and VLAIP containing material from CM-cellulose (0.05 ml, 30 mg/ml in 0.1 M ammonium acetate pH 8.0) was injected into the TSK-DEAE column (0.8 × 15 cm). The flow rate was kept constant at 1 ml/min. The elution protocol was as follows: a linear gradient of 0.1 M ammonium acetate pH 8.0 to 1 M ammonium acetate pH 8.0 was run over 25 min; the run was completed by isocratic elution with 1 M ammonium acetate for 10 min. The fractions were collected manually. The first fraction containing VLAIP was rechromatographed at the same conditions.

#### 2.2.4.4. Chromatofocusing on PBE-94 column

Four milliliters of start buffer containing 21 mg of VLFXA (CM-cellulose fraction) were used for chromatofocusing on the PBE94 column (0.5 × 30 cm). Elution conditions were: start buffer, 0.025 M imidazole–HCl, pH 7.4; elution buffer, polybuffer 74 (1:8) pH 4.0; flow rate 13 ml/h; fraction volume 3 ml. The elution was performed with polybuffer in the interval of pH 7–4. Absorbance was continuously monitored at 280 nm and the pH and coagulant activity of fractions were measured.

### 2.2.5. Protein determination

Protein concentrations were determined using the Pierce micro BCA kit. Bovine serum albumin was used as standard for detecting the protein concentration. The method is based on the reaction of protein with $Cu^{2+}$ to form $Cu^{+}$, which complexes with bicinchoninic acid (BCA), forming a colored product. The intensity of this product at 562 nm is related to protein concentration.

### 2.2.6. SDS–PAGE

SDS–polyacrylamide gel electrophoresis was carried out in 10% and 12.5% gels at pH 8.3 by the method of Laemmli (1970). The following molecular mass indicators were used: bovine serum albumin (67 kDa), ovalbumin (43 kDa), bovine carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and cytochrome C (12.3 kDa). Protein staining was performed with Coomassie brilliant blue R 250. Schiff reagent was used for glycoprotein staining.

### 2.2.7. In-gel tryptic digestion

After visualization with Coomassie Blue the gel-electrophoresis bands of protein in interest (reduced or non-reduced) were excised from SDS-polyacrylamide gels, each gel slice was cut into small pieces (1 $mm^2$), placed into Eppendorf tubes and completely destained with 50 mM $NH_4HCO_3$ in 40% EtOH. The unstained gel pieces were washed with 200 µl of 25 mM $NH_4HCO_3$ three times for 15 min with vortexing, dehydrated with 100 µl of acetonitrile (ACN) three times for 10 min with vortexing and lyophilisized to complete dryness. For alkylation of cysteins 50 µl of 50 mM iodoacetamide in 25 mM $NH_4HCO_3$ was added to dried gel pieces and the reaction was allowed to proceed for 45 min in the dark at room temperature. The supernatant was discarded and gel pieces were washed with 100 µl of 100 mM $NH_4HCO_3$ for 15 min with vortexing, dehydrated with 200 µl of ACN two times for 15 minutes with vortexing, rehydrated by swelling in 100 µl of 100 mM $NH_4HCO_3$ for 15 min with vortexing, dehydrated with 200 µl of ACN two times for 15 minutes with vortexing and lyophilisized.

Tryptic digestion was started with the addition of 5 µl of 75 ng/µl trypsin (Promega) solution in 25 mM $NH_4HCO_3$ to the dried gel pieces. After reswelling the gel pieces were covered with an overlay of ~25 µl 25 mM $NH_4HCO_3$ so that the gel pieces remained immersed throughout the digestion. The proteins were digested 20 h at 37 ºC.

After collection of the overlays into clean Eppendorf tubes 30 µl of extraction solution (60% ACN, 1% TFA, 0.1% n-octylglucoside) was added to the gel pieces and vortexed for 45 minutes, the extracts were added to the initial overlays. The extraction was repeated once more. The extracts were dried with SpeedVac.

### 2.2.8. MALDI-TOF mass spectrometry of proteins and their tryptic fragments

The MALDI mass spectra of VLFXA, human factor X, VLAIP, cleavage products of human factor X and tryptic peptides of VLFXA and VLAIP were measured with a home-built MALDI mass spectrometer designed for maximum flexibility in use (National Institute of Chemical Physics and Biophysics, J.Subbi). Peptide (human factor X and IX fragments) solutions were directly prepared in 0.1 M $NH_4HCO_3$, in

concentrations of about 1 mg/ml, and kept frozen at −20 °C until use. The enzymatic hydrolysis of peptides was carried out in 0.1 M $NH_4HCO_3$, at 37 °C in an Eppendorf tube. In a typical experiment, 100 µl of 0.1 M $NH_4HCO_3$ solution of substrate (1 mg/ml) in an Eppendorf tube was thermally equilibrated to 37 °C in the thermostated rack. The reaction was started by addition of 15 µl of VLFXA solution (1 mg/ml in 0.1 M $NH_4HCO_3$). At predetermined time intervals (5 min, 0.5 h, 20 h), the aliquot (10 µl) was diluted with 100 µl of $H_2O$ and 2 µl of 6 N HCl were added to stop the reaction. We applied a high enzyme–substrate ratio (1:7 on a weight basis) to determine the position of cleavage. One half microliter of diluted mixture was used for MALDI-TOF mass spectrometry analysis. The matrix used for peptide analyses was 2,5-dihydroxybenzoic acid (DHB). Ten milligrams of DHB were dissolved in 1 ml of a 1:1 mixture of 0.1% trifluoroacetic acid and acetonitrile for sample preparation. Half a microliter of this mixture was deposited on a stainless steel probe tip, mixed there with 0.5 µl of reaction mixture of peptides and VLFXA and allowed to dry at room temperature. External mass calibration was accomplished using substance P and Lys-bradykinin as peptide standards.

For MALDI-TOF analysis the dried samples were dissolved in 5 µl of 50% ACN, 0.1% TFA. Aliquots of 0.5 µl were applied onto the target, allowed to air dry and 0.5 µl of the matrix solution (2,5-dihydroxybenzoic acid - DHB or α-cyano-4-hydroxycinnamic acid-CHCH) was applied to the target and allowed to dry in air. Before MALDI-TOF analyses in some cases protein samples were purified from salts using ZipTip $C_4$ or ZipTip $C_{18}$ according to Millipore instructions. The matrix used for MALDI-TOF MS analyses of proteases, factor X and cleavage products of factor X was ferulic acid. Cytochrome C and bovine carbonic anhydrase were used for mass calibration. The external mass calibration was accomplished by using substance P and bradykinin as peptide standards.

### 2.2.9. Electrospray tandem mass spectrometry

Electrospray tandem mass spectrometry experiments were performed at the Protein Facility of the Institute of Biotechnology, University of Helsinki. Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-MS/MS) was performed on a Q-TOF hybrid mass spectrometer (Micromass Ltd., Manchester, UK) connected to an Ultimate nano liquid chromatograph (LCPackings, The Netherlands).

### 2.2.10. Proteolytic activity assays

Azocasein was synthesized by diazotization of casein as described by Charney and Tomarelli (1947). Azocaseinolytic activity was measured as described by Siigur et al. (1998b).

VLAIP-dependent digestion of fibrinogen was assessed with bovine fibrinogen as substrate. 0.1 ml of 2% fibrinogen solution was incubated with 0.1 ml of VLAIP (10 µg) at 37 ºC in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl. At various time intervals 0.025 ml of the incubation mixture was withdrawn and added to 0.025 ml of denaturing solution (10 M urea, 4% SDS, 4% 2-mercaptoethanol). The samples were incubated overnight at 37 ºC before electrophoresis. Specific cleavage of fibrinogen was shown on 12.5% SDS-polyacrylamide gels.

Gelatinolytic activity was determined according to Bee et al. (2001). Gelatin was co-polymerised into 10% PAA-gel at final concentration of 2.5 mg/ml. VLAIP (5 µg) was mixed with non-reducing sample buffer and applied to the gel. After

electrophoresis the SDS was removed by washing the gel for 1 h in 2.5% Triton X-100 before incubation in 50 mM Tris-buffer (50 mM $CaCl_2$, 0.2 M NaCl, 0.07% Brij 35), pH 7.6 at 37 $^{o}$C for 16 h and staining with 0.125% Coomassie brilliant blue R250. Clear areas in the gel indicate regions of enzyme activity.

**2.2.11 Experiments with cells: adhesion assays and detection of apoptosis were carried out in cooperation with Priit Kogerman group and are described in detail in the paper VI.**

## 3. RESULTS AND DISCUSSION

### 3.1. Isolation of *V. lebetina* venom metalloproteinases (II, VI)

Purification of the factor X activator (VLFXA) and VLAIP was achieved by a three-step procedure. The crude venom was initially fractionated by gel filtration in a column of Sephadex G-100 (sf), ten fractions were collected (Fig. 2).

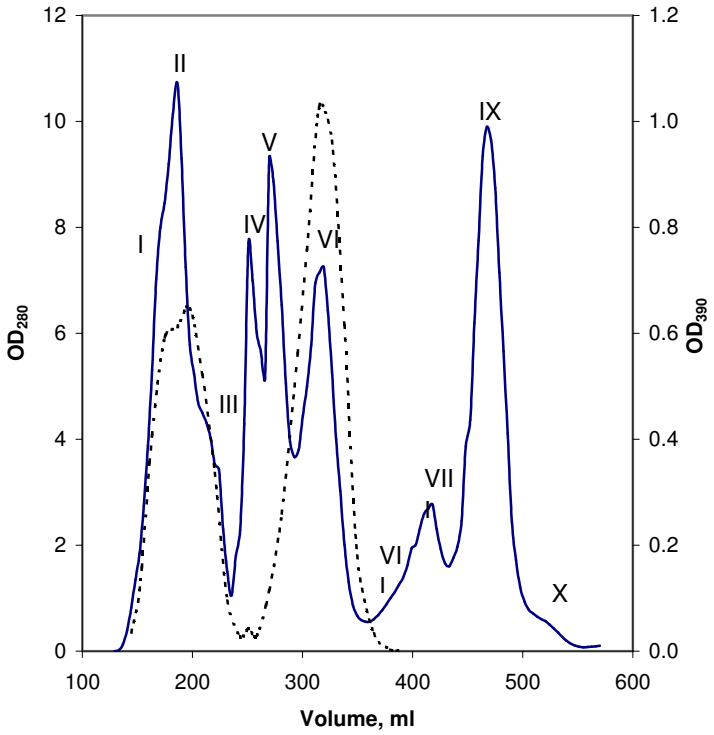



Fig. 2. Gel filtration of *V. Lebetina* venom. Crude venom (1.5 g) was dissolved in 15 ml of 0.2 M ammonium acetate, pH 6.7. Insoluble material was removed by centrifugation (5000 x g for 15 min) and the supernatant was applied to the column (2.8 x 128 cm) of Sephadex G-100 superfine equilibrated with 0.2 M ammonium acetate. The elution was carried out with the same solution at a flow rate of 3.8 ml/h and fractions were collected at 1 h intervals. Protein concentration was monitored at 280 nm (——), azocaseinolytic activity was monitored by measuring absorbance at 390 nm (·····).

The blood clotting and factor X activating activity was found in the first and second peaks. These peaks also contain L-amino acid oxidase, proteinases, 5′-nucleotidase, phosphodiesterase, phosphomonoesterase and the endothelial cells affecting component as well. In this stage of purification FXA and VLAIP were separated from metalloprotease lebetase (in the sixth fraction) (Siigur and Siigur 1991) and from serine proteases: factor V activator (fourth fraction, Siigur et al 1998a), bradykinin releasing enzymes (fourth fraction, Siigur et al 1982) and fibrinogenases (Siigur et al 1991,

2003). Cation exchange chromatographies in CM-cellulose and anion exchange in TSK-DEAE HPLC were performed for further purification. In these purification steps VLFXA and VLAIP were separated from other high molecular mass proteinases, nucleases and LAO. The fractionation of VLFXA containing material, obtained from different venom batches by gel filtration, on CM-cellulose at pH 5.5 (Fig.3) yielded several components that activated human and bovine factor X, which eluted at different ionic strengths. The factor X activators present in the II asymmetrical peak were further purified on TSK-DEAE HPLC column (Fig. 4). Three protein peaks were obtained from which VLAIP was found in the first peak and the procoagulant and factor X activation activities were recovered in the last two peaks. Additional rechromatography on TSK-DEAE HPLC in same conditions of the first protein peak was used for VLAIP. Chromatofocusing of fraction II from CM-cellulose revealed two main VLFXA peaks with p*I* values of 5.9 and 5.6 (Fig. 5). The average yields of FXA and VLAIP from 1.5 g of crude venom were about 45 mg and 3 mg respectevely.

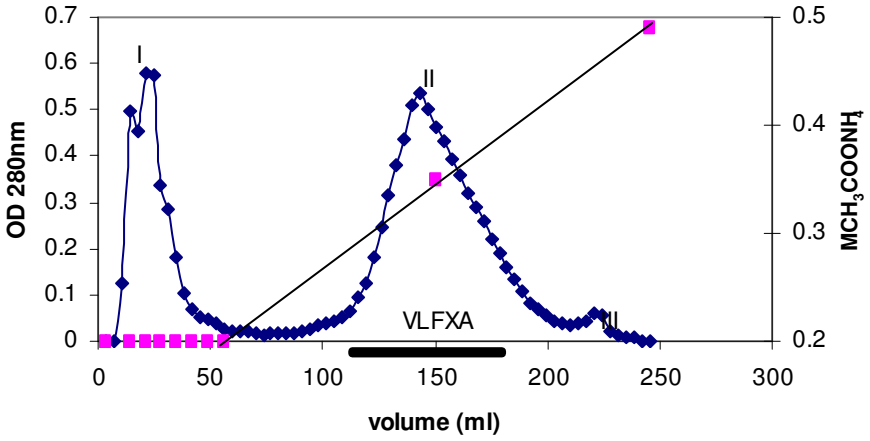



Fig. 3. Chromatography of fraction I–II (Fig. 2) on CM-cellulose. The column (1.8×10 cm) of CM cellulose was equilibrated with 0.2 M ammonium acetate, pH 5.5. 30 mg of lyophilized fraction I–II was dissolved in 5 ml of 0.2 M ammonium acetate pH 5.5 and applied to the column. Non-adsorbed material was eluted with the same solution. The adsorbed material containing factor X activating enzyme and VLAIP was eluted with ammonium acetate gradient 0.2–0.5 M, pH 5.5, flow rate 12 ml/h; fractions of 3 ml were collected.

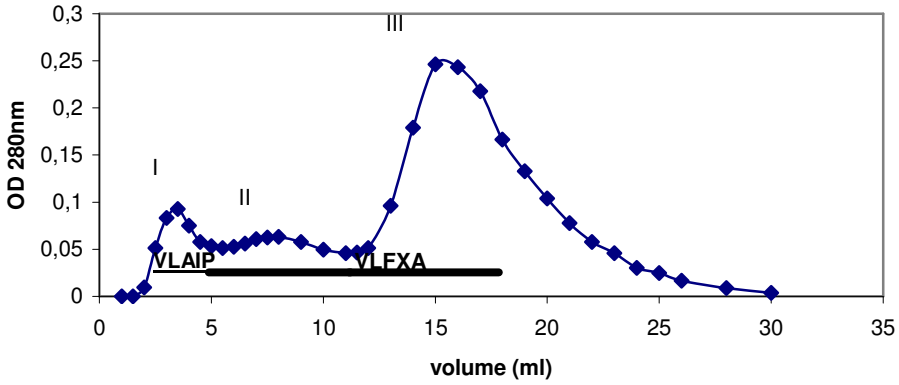



Fig. 4. Chromatography of VLFXA fraction from CM-cellulose on TSK-DEAE HPLC. The results of an example run are shown. VLFXA and VLAIP containing material from CM-cellulose (0.05 ml, 30 mg/ml in 0.1 M ammonium acetate pH 8.0) was injected into the TSK-DEAE column (0.8 × 15 cm). The flow rate was kept constant at 1 ml/min. The elution protocol was as follows: a linear gradient of 0.1 M ammonium acetate pH 8.0 to 1 M ammonium acetate pH 8.0 was run over 25 min; the run was completed by isocratic elution with 1 M ammonium acetate for 10 min.

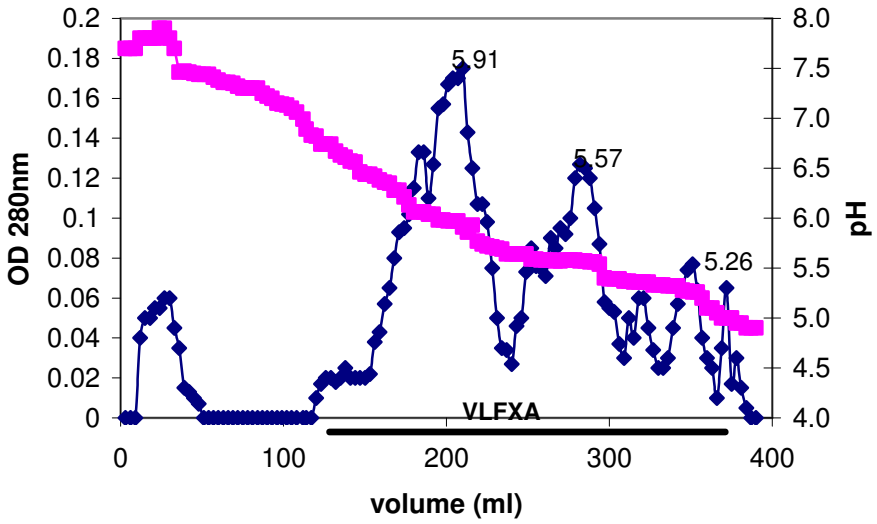



Fig. 5. Chromatofocusing of VLFXA preparation from CM-cellulose on PBE 94. Column: 0.6×38 cm. 21 mg of VLFXA fraction from CM-cellulose were dissolved in 4 ml of start buffer. Elution conditions: start buffer 0.025 M imidazole–HCl, pH 7.4, elution buffer pH 4.0, polybuffer 74–HCl (dilution 1/8), flow rate 13 ml/h, fraction volume 3 ml. ♦, protein fractions detected by absorbance at 280 nm; ---- pH gradient.

The specific activity of the purified VLFXA was about 6 times higher than that of crude venom. A prothrombin time of 12 s was achieved with 5 μg of the VLFXA preparation. VLFXA did not act on fibrinogen and on prothrombin, and had no arginine esterase activity. Different venom batches showed different levels of factor X activation and the yield of purification was variable.

### 3.2. Characterization of VLFXA and VLAIP (II, V, VI)

The preparations of the VLFXA and VLAIP were analyzed by MALDI-TOF MS and by SDS–PAGE. The purified reduced FXA has a heavy chain (HC) of 57.5 kDa and two light chains 17.4 kDa (LC1) and 14.5 kDa (LC2) (SDS–PAGE) (Fig.6). The sugar content of VLFXA was about 15.5% (8% hexoses, 5% hexosamines, 2.5% neuraminic acid) for the purified VLFXA. Gowda showed that the oligosaccharide chains are necessary for the functional activity of RVV-X (Gowda et al 1996).

The N-terminal sequence of the heavy chain of VLFXA determined from the protein is 100% identical to the sequence deduced from the cDNA. Molecular masses of tryptic fragments of VLFXA HC and the sequences of the internal peptide fragments detected by LC-MS/MS also confirm the protein sequence deduced from the cDNA. There are three potential N-glycosylation sites in the deduced sequence. However, it is still not clear, are all these sites ($Asn^{259}$, $Asn^{353}$ and $Asn^{373}$) linked with carbohydrates (Fig. 9).

The N-terminal sequences of LC1 and LC2 determined from the protein sequencing as well as the inner sequence fragment determined from the protein sequencing are 100% identical to the sequences deduced from the cDNAs. The protein sequence of LC1 includes one potential N-glycosylation site at $Asn^{47}$. There are no potential N-glycosylation sites in the protein sequence of LC2. The molecular masses and sequences of tryptic fragments of VLFXA LC1 and LC2 confirm the protein sequences deduced from the cDNAs (Fig. 10 and 11). VLFXA is an example of a P-IV class protein (see Fig. 1, p. 11) that contains proteinase domain, disintegrin-like domain, cysteine-rich domain and lectin-like domains that are synthesized from three different genes.

Isoelectric focusing studies revealed heterogeneity of the enzyme. It gave several poorly resolved bands in the pH range of 5.3–6.6. Isoelectric heterogeneity of enzymes appears to be related to their sialic acid content. After neuraminidase treatment the distribution of isoelectric points was different from that before treatment (data not shown) suggesting that VLFXA exists in multiple isoforms; the differences in isoelectric points were due, at least in part, to dissimilarities of the respective sialic acid content of isoforms. No functional differences were, however, discovered between any of the isoforms purified from different batches of venom. Data about isoforms of RVV-X are contradictory; most authors have found one isoform, only Amphlett et al (1982) isolated multiple subforms of enzyme. The factor X activating enzyme is thermolabile. The activity of VLFXA gradually decreases after heating beginning from 50 °C. On heating at 70 °C for 10 min about 55% of the factor X activating activity was lost.

Molecular masses of different VLFXA samples detected by MALDI-TOF analyses were in the interval of 85–91 kDa. Most purified factor X activators from different snake venoms have high molecular masses: 92.88 kDa from *V. russelli* venom (Gowda et al 1994), 79 kDa from *V. russelli* venom (Takeya et al 1992), 75 kDa from *B. atrox* venom (Hofmann and Bon 1987), and 76 kDa from *C. cerastes* venom (Franssen et al 1983). There are contradictory data regarding the molecular masses and polypeptide chain compositions of *V. russelli* venom factor X activator (Kisiel et al 1976, Takeya et

al 1992, Gowda et al 1994 and Gowda et al 1996). RVV-X consists of three disulfide linked glycopolypeptide chains: one heavy chain (α-chain, 57.6 kDa) and two light chains (β- and γ-chains, 19.4 kDa and 16.4 kDa, respectively) (Gowda et al 1994). Low molecular mass factor X activators were purified from *C. cerastes* venom (El-Asmar et al 1986) and *C. vipera* venom (Farid et al 1993) (14 kDa and 12.5 kDa, respectively). *Vipera berus berus* factor X activating enzyme is different from all of them, having a molecular mass of 38 kDa (Samel and Siigur 1995).

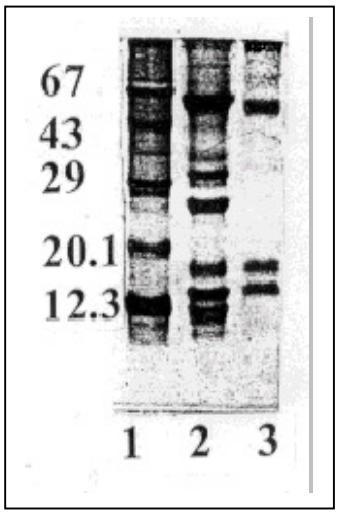



Fig. 6. SDS–PAGE of VLFXA in 12.5% gels. Lanes: 1, standard proteins were bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and cytochrome *c* (12.3 kDa); 2, *V. lebetina* venom; 3, VLFXA.

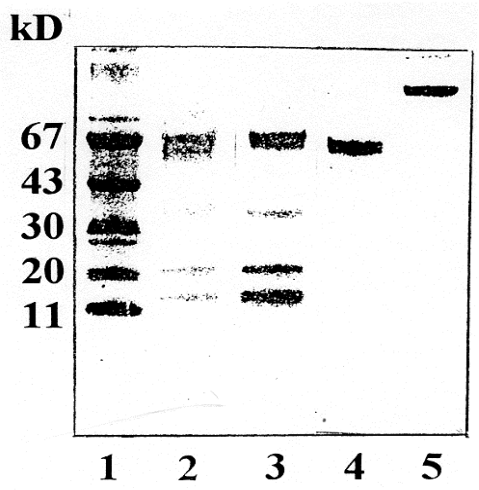



Fig. 7. Tricine SDS-PAGE in 12.5% gels. Lane 1, molecular weight markers; lane 2, fraction II from Sephadex G-100 sf.; lane 3, fraction II from CM-52 cellulose; lane 4, fraction I (VLAIP) from TSK-DEAE HPLC (under reducing conditions); lane 5, fraction I (non-reduced).

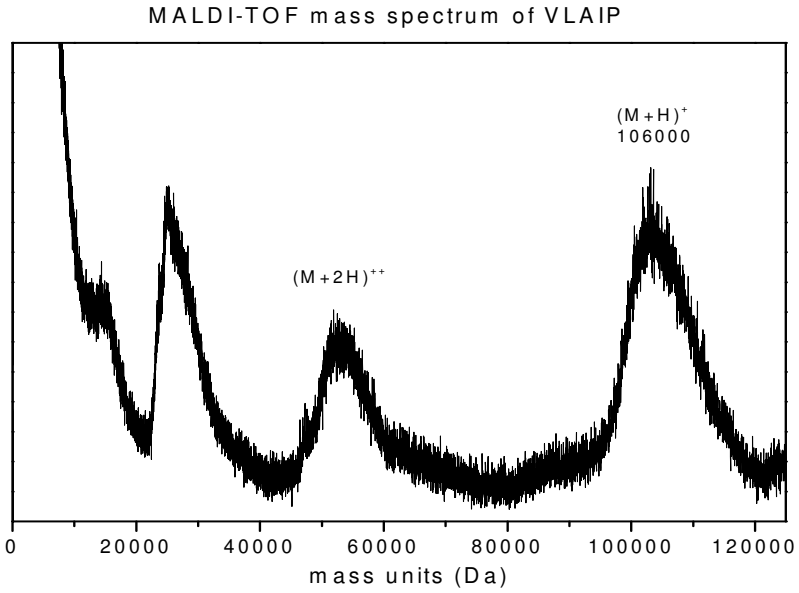



Fig. 8. MALDI-TOF mass spectrum of VLAIP.

Purified VLAIP showed a molecular mass of about 60 kDa on SDS-PAGE analysis under reducing conditions (Fig. 7) and 106 kDa on MALDI-TOF MS analyses (Fig. 8).

The direct sequence analysis of tryptic fragments of VLAIP revealed fragments belonging to VLAIP-A and VLAIP-B. The sequences of the internal peptide fragments are in concordance with amino acid sequences deduced from the cDNA. Staining by the periodic acid/Schiff procedure (PAS staining) indicated that the purified VLAIP is glycoprotein. There is one potential N-glycosylation site in VLAIP-A and four in VLAIP-B (Fig. 9). However, it remains to be identified whether all these sites are linked with carbohydrates.

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 (Grams et al 1993). Direct N-terminal sequencing of VLAIP resulted in no sequence, suggesting that the amino terminus of the protein is blocked. Many snake venom metalloproteases have been reported to contain pyroglutamate as the amino terminal amino acid (Siigur et al 1998b). The deduced amino acid sequences of both forms have a typical $Zn^{2+}$-chelating sequence **H-E-X-X-H-X-X-G-X-X-H-D** in the catalytic domain that is conserved in snake venom metalloproteases (Bjarnason and Fox 1995). Proceeding from the conserved intrachain disulfide bond pattern of 6-Cys metalloproteases, the S-S bridges in VLAIP chains should look as follows: $Cys^{314}$-$Cys^{394}$, $Cys^{354}$-$Cys^{378}$ and $Cys^{356}$–$Cys^{361}$. $Cys^{369}$ remains free and could be used for formation of interchain disulfide bond between the two monomers. $Cys^{369}$ seems to be typical for vascular apoptosis inducing proteases, many other snake venom metalloproteases have different amino acids in this position (Fig. 9). The disintegrin-like domain contains ECD motif that is characteristic for many P-III type reprolysins (Kishimoto et al 2002, Silva et

al 2003, Siigur et al 2004) and also for ADAM metalloproteases (Ceretti et al 1999). The regions of ECD motif are identical for both VLAIPs (see Fig. 9), this region might be essential for binding to integrins.

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VLAIP A                 MMQVLLVTISLAVFPYQGSSIILESGNVNDYEVVYPQKVTAMPKGAVKQP
VLAIP B                 MMQVLLVTICLAVFPYQGSSIILESGNVNDYEVVYPQKITALPKGAIQQP
factor X activator HC   MMQVLLVTISLAVFPYQGSSIILESGNVNDYEVVYPQKITALPEEAVQQP
                        *:*******:**********::*:**********:**::*::*::*::**
                        <signal sequence >< prepropeptide

                        51                                                100
VLAIP A                 EQKYEDAMQYEFKVKGEPVVLLLEKNKDLFSEDYSETHYSPDGREITTNP
VLAIP B                 EQKYEDAIKYEFKVNGKPVVLHLEKNKGLFSEDYSETHYTPDGREITINP
factor X activator HC   EQKYEDTMQYEFEVNGEPVVLHLEKNKDLFSEDYSETRYSPDGRETTTKP
                        *:****:::***:*:*:***::****::***:*****:*:*****:*::*

                        101                                               150
VLAIP A                 PVEDHCYYHGRIQNDADSSASISACNGLKGHFMLQGETYLIEPLKLPDSE
VLAIP B                 PVEDHCYYHGRIQNDADSTASISACNGLKGHFKLQGEMYLIEPLRIPDSE
factor X activator HC   PVQDHCYYHGRIQNDAYSSASISACNGLKGHFKLQGETYLIEPLKIPDSE
                        :*:*************:*:***********:*:*:**:******::****

                        151                                               200
VLAIP A                 AHAVYKYENVEKEDEAPKMCGVTQTNWESDEPIKKASQLNLTPEQRRYLN
VLAIP B                 AHAIYKYENIEKEDEAPKMCGVTQTNWESDEPIK-ASQLNLTPEQRTYLK
factor X activator HC   AHAVYKYENIEKEDEAPKMCGVTQTNWESDEPIKKASQLVATSAKRKFHK
                        ***:*****:*****************:**:::*:****::*:::::::::
                                                              ><

                        201                                               250
VLAIP A                 SPKYIKLVIVADYIMFLKYGRSLITIRTRIYEIVNILNVIYRVLNIYIAL
VLAIP B                 SKKYVELVIVADYIMFWKYDRSLSTIRTRIYEIVNTLNVIYRFLNIYIAL
factor X activator HC   T--FIELVIVVDHRVVKKYDS-AATNTKIYEIVNTVNEIFIPLNIRLTL
                        ::::::****:*:::::*:::::::::*:::::**:::::::::**:::::*
                                metalloproteinase

                        251                                               300
VLAIP A                 LGLEIWNNGDKINVLPETKVTLDLFGKWRERDLLNRRKHDNAQLLTDINF
VLAIP B                 VAVEIWSKGDLINVTSSAYDTLDSFGEWRERDLLNRKRHDNAQLLTGINF
factor X activator HC   IGVEFWCNRDLINVTSSADDTLDSFGEWRGSDLLNRKRHDNAQLFTDMKF
                        :::*:*::::*:***::::::::*::*****::*****::**:***:*::::

                        301                                               350
VLAIP A                 NGPTAGLGYVGSMCDPQYSAGIVQDHNKVNFLVALAMAHEMGHNLGMEHD
VLAIP B                 NGPSAGRGFVGRMCQPKYSVGIVQDHSKIYLLVASAMAHEMGHNLGMDHD
factor X activator HC   DLSTLGITFLDGMCQAYRSVGIVQEHGNKNFKTAVIMAHELGHNLGMYHD
                        :::::*:::::::**:::::*:****:*::::::::*::****:******:**

                        351                                               400
VLAIP A                 EIHCTCGAKSCIMSGTLSCEASIRFSNCSREEHQKYLINKMPQCILNKPL
VLAIP B                 RIDCTCGAKSCIMSGILRCETSYLFSDCSREEHRKYLINKMPQCILNKPL
factor X activator HC   RKNCICNDSSCIMSAVLSSQPSKLFSNCSNHDYRRYLTTYKPKCILNPPL
                        :::*:*::::*:**:::::::*::**:**::::::**::::*:****:*:
                                                                    ><
```

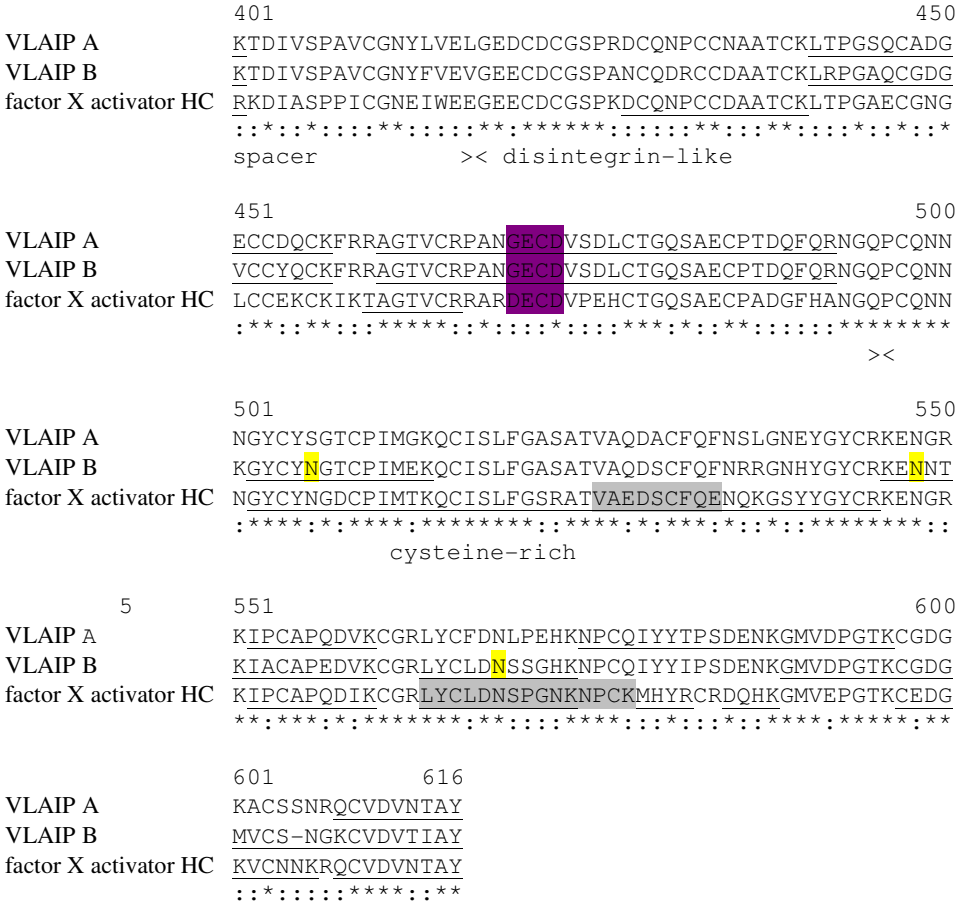



Figure 9. Comparison of protein sequences of metalloproteinases (deduced from cDNA) of the current study. Identical amino acids at the same position are marked by "*", different amino acids by ":". The highlighted sequences are following: cysteine switch motif is in a blue box, Zn-chelating motif is in a red box, xECD motif is in a lilac box; the potential N-glycosylation sites are yellow. The peptide fragment sequences detected by LC-MS/MS are in gray boxes. Tryptic peptide fragments detected by MALDI-TOF MS (mass tolerance less than 0.5 Da) are underlined.

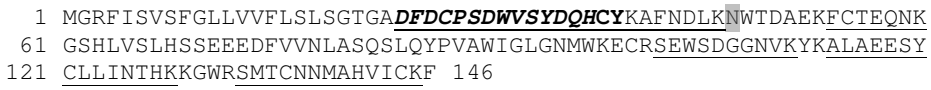



Fig. 10. Complete amino acid sequence (deduced from cDNA) of the light chain 1 of VLFXA. The amino acid sequence is numbered from the N-terminal amino acid of the preproprotein. The N-terminus detected from protein sequence is bold italics. Tryptic peptide fragments detected by MALDI-TOF MS (mass tolerance less than 0.5 Da) are underlined. The potential site of N-glycosylation (Asn47) is grey.

```
  1 MGRSISVSFGLLAVFLSLSGTG***GLDCPPDSS***PRYFCYRVFKEQKNWADAERFCAERPNNG
 61 HLVSIESMEEAEFVAQLLSKITGKFITHFWIGLRIEDKKQQCRSE**WSDGSSVSYD**NLLKR
121 EFRKCFGLEKGTGYRSWFNLNCEEPYPFVCKVPPNC 158
```

Fig. 11. Complete amino acid sequence (deduced from cDNA) of the light chain 2 of VLFXA precursor. The amino acid sequence is numbered from the N- terminal amino acid of the preproprotein. The N-terminus detected from protein sequence is bold italics. The peptide fragment sequences detected by LC-MS/MS are grey boxed. Tryptic peptide fragments detected by MALDI-TOF MS (mass tolerance less than 0.5 Da) are underlined.

### 3.3. Substrate specificity

Specificity of *V.lebetina* venom metalloproteases was studied against different proteins and peptides.

#### 3.3.1. Hydrolysis of peptides (I, II, IV)

Lebetase hydrolyzes a variety of bioactive peptides as monitored by MALDI-TOF mass spectrometry with the results summarized in table 2.

Table 2. MALDI–TOF analysis of reaction mixture after hydrolysis of substrate by lebetase I.

| No | Substrate P1↓P1´ | ($M_w$+H) | | Fragments | ($M_w$+H) | |
|---|---|---|---|---|---|---|
| | | Calc. | MALDI | | Calc. | MALDI |
| 1 | Substance P<br>RP↓KP↓QQFFG↓LM | 1347.73 | 1347. | RPKP<br>QQFFGLM<br>KPQQFFGLM<br>QQFFG<br>RP | 497.30<br>870.40<br>1095.55<br>626.28<br>271.30 | 497.9<br>891.8[a]<br>1117.5[a]<br>648.7[a]<br>-[c] |
| 2 | Bradykinin<br>RPPGFSP↓FR | 1060.55 | 1061.2 | RPPGFSP<br>FR | 757.39<br>322.17 | 758.2<br>322.3 |
| 3 | Lys-bradykinin<br>KRPPGFSP↓FR | 1188.65 | 1189.8 | KRPPGFSP<br>FR | 885.48<br>322.17 | 886.3<br>322.3 |
| 4 | Glycagon<br>HSQGTFTSDY↓SK<br>YLDSRRAQ-<br>DFVQWLMNT | 3483.76 | 3481.6 | HSQGTFTSDY<br>SKYLDSRRAQ-<br>DFVQWLMNT | 1142.46<br>2359.64 | 1142.8<br>2357.6 |
| 5 | YVP↓QLGT | 777.40 | 777.4 | YVP<br>QLGT | 378.18<br>418.21 | 378.1<br>418.2 |
| 6 | PYVP↓QLGT | 874.45 | 873..9 | PYVP<br>QLGT | 475.24<br>418.21 | 474.4<br>418.3 |
| 7 | PEG↓LRVGF | 874.45 | 874.7 | PEG<br>LRVGF | 302.12<br>591.34 | -[d]<br>591.6 |
| 8 | GPEG↓LRVGF | 931.48 | 931.7 | GPEG<br>LRVGF | 359.14<br>591.34 | 381.2[a]<br>591.6 |
| 9 | GHAR↓LVHV | 888.50 | 888.2 | GHAR<br>LVHV | 440.22<br>467.28 | 440.0<br>467.1 |
| 10 | FSP↓MLGE | 796.34[b] | 796.6[b] | FSP<br>MLGE | 350.15<br>449.19 | 350.2<br>465.3[b] |
| 11 | FFSP↓MLGE | 943.41[b] | 944.6[b] | FFSP<br>MLGE | 497.22<br>449.19 | 497.8<br>465.7[b] |
| 12 | EYHTEK↓LVTS | 1206.58 | 1206.3 | EYHTEK<br>LVTS | 806.35<br>419.23 | 806.6<br>419.3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 13 | YHTEK↓LVTS | 1077.54 | 1077.8 | YHTEK<br>LVTS | 677.31<br>419.23 | 677.5<br>419.3 |
| 14 | HTEK↓LVTS | 914.48 | 914.9 | HTEK<br>LVTS | 514.24<br>419.23 | 514.6<br>419.4 |
| 15 | VPQLGT | 614.33 | 614.7 | no cleavage | | |
| 16 | SPMLGE | 633.27 | 633.3 | no cleavage | | |
| 17 | GLRVGF | 648.36 | 648.8 | no cleavage | | |
| 18 | ARLVHV | 694.42 | 695.0 | no cleavage | | |
| 19 | HAR↓LVHV | 831.48 | 832.3 | HAR<br>LVHV | 382.41<br>467.28 | 383.2<br>467.3 |

↓, the cleavage site of peptide by lebetase; **↓**, arrow corresponds to the main cleavage site (after 5 min hydrolysis) of substance P by lebetase I.

[a] $(M_w + Na)^+$.

[b] Observed masses corresponding to peptides containing an oxidized Met residue (+16 Da).

[c] Due to matrix background RP was not determined

[d] Due to matrix background PEG was not determined.

[e] Cleavage was not seen only after 24 h incubation with lebetase I.

Lebetase cleaved peptides containing seven or more amino acid residues, hexapeptides were not hydrolyzed (Table 2, peptides 15–18)

The hydrolysis of oxidized insulin B-chain has been described extensively for assessment of proteolytic specificity. Detailed cleavage analysis of insulin B-chain and its fragments by snake venom hemorrhagic metalloproteinases is provided in literature (Bjarnason and Fox 1995, Bjarnason and Fox 1994, Fox et al 1986). Metalloproteinases from snake venoms mainly split the insulin B-chain at Leu and/or Phe residues at positions $P_1'$ (N-terminal cleavage) (Bjarnason and Fox 1995, Bjarnason and Fox 1994). In previous studies (Samel and Siigur 1990, 1995, Siigur and Siigur 1991) amino acid analysis of proteolysis cleavage products of insulin B-chain has been used in our laboratory. The cleavage of insulin B-chain by lebetase and VLAIP was investigated using MALDI-TOF MS that is more effective and quick method for detection of peptide fragments. Lebetase (Siigur and Siigur 1991) cleaves $Ala^{14}$- $Leu^{15}$ and $Tyr^{16}$-$Leu^{17}$ bonds of oxidized insulin B-chain (Table 2). Recently isolated nonhemorrhagic fibrinolytic enzyme leucurolysin-a from *Bothrops leucurus* snake venom cleaves the same bonds in oxidized insulin B-chain as lebetase (Bello et al 2006). Differently from lebetase VLAIP cleaved only $Ala^{14}$-$Leu^{15}$ bond in oxidized insulin B-chain. VLFXA does not hydrolyze insulin B-chain at all.

Table 3. Comparison of hydrolysis of peptide substrates by lebetase and VLAIP

| **Name of substrate** | **Lebetase cleavage sites** | **VLAIP cleavage sites** |
|---|---|---|
| Oxidized insulin B chain<br>FVNQHLC($SO_3$H)GSHLVEA$^{14}$L$^{15}$-<br>Y$^{16}$L$^{17}$VC($SO_3$H)GERGFFYTPKA | $Ala^{14}$-$Leu^{15}$<br>$Tyr^{16}$-$Leu^{17}$ | $Ala^{14}$-$Leu^{15}$ |
| Substance P<br>RPKPQQFFGLM | ***$Pro^4$-$Gln^5$**<br>$Gly^9$-$Leu^{10}$<br>$Pro^2$-$Lys^3$ | $Gln^5$-$Gln^6$<br>$Phe^7$-$Phe^8$<br>$Gly^9$-$Leu^{10}$ |
| Bradykinin<br>RPPGFSP↓FR | $Pro^7$-$Phe^8$ | $Pro^7$-$Phe^8$ |
| Fibrinogen fragment 406-417 | | |

| | | |
|---|---|---|
| HTEK↓LVTS | $Lys^4$-$Leu^5$ | $Lys^4$-$Leu^5$ |
| Fibrinogen fragment 513-520<br>FFSP↓MLGE | $Pro^4$-$Met^5$ | $Pro^4$-$Met^5$ |
| $\alpha_2$-M fragment 693-700<br>GHAR↓LVHV | $Arg^4$-$Leu^5$ | $Arg^4$-$Leu^5$ |
| $\alpha_2$-M fragment 676-684<br>GPEG↓LRVGF | $Gly^4$-$Leu^5$ | $Gly^4$-$Leu^5$ |
| PZP fragment 686-693<br>PYVP↓QLGT | $Pro^5$-$Gln^6$ | $Pro^5$-$Gln^6$ |

***Pro$^4$-Gln$^5$** is the main cleavage site (after 5 min hydrolysis) of substance P by lebetase.

Substance P was cleaved by lebetase mainly at $Pro^4$-$Gln^5$ position. After 20 h of incubation, substance P was totally hydrolyzed to peptide fragments. Slow hydrolysis was also detected at positions $Pro^2$–$Lys^3$ and $Gly^9$–$Leu^{10}$. The cleavage of substance P by VLAIP is rather different (Table 3). ECE-1 (Johnson et al 1999) and human matrix metalloproteinase-9 (Backstrom and Tokes 1995) did not cleave substance P at $Pro^4$–$Gln^5$ position, their main cleavage sites were $Gln^6$–$Phe^7$ and $Gly^9$–$Leu^{10}$. Substance P seems to be a good substrate for differentiating specificity of the metalloproteinases.

Many snake venom proteases have fibrinogenolytic activities. Human fibrinogen and fibrin have often been used as substrates for various venom proteinases (reviews Bjarnason and Fox 1994, 1995, Siigur and Siigur 1992 and Markland 1998). Several fibrinogen cleavage sites on the Aα-chain have been identified for fibrinolytic enzymes; fibrolase from *Agkistrodon contortrix contortrix* venom cleaves at $Lys^{413}$–$Leu^{414}$ (Retzios 1992); fibrinolytic enzymes from *Crotalus basiliscus* venom Cbfib 1.1 and Cbfib 1.2 cleave at $Lys^{413}$–$Leu^{414}$, $Ser^{505}$–$Thr^{506}$, $Tyr^{560}$–$Ser^{561}$; Cbfib 2 cleaves mainly at $Gly^{254}$–$Ser^{255}$, $Pro^{516}$–$Met^{517}$ (Retzios 1994). *V. lebetina* venom metalloproteases (lebetase, VLAIP) cleave mainly Aα-chain of fibrinogen at different positions and more slowly Bβ-chain. *V. lebetina* venom serine proteinase - α-fibrinogenase degrades only Aα-chain (Siigur et al 1991, Samel et al 2002). None of them attacks γ-chain of fibrinogen. *V. lebetina* venom also contains thermostable serine proteinase - β-fibrinogenase - that preferably cleaves β-chain of fibrinogen (Samel et al 2002). Previously it was shown that cleavage patterns of fibrinogen treated with venom proteases are different (Samel et al 2002, Siigur and Siigur 1991). Preliminary molecular weights of cleavage products were detected by SDS-PAGE analysis. Judged from the degradation products, the cleavage specificity of fibrinogen for lebetase is broader than that for fibrolase (Siigur et al 1998). We synthesized some 6–10 amino acid residues containing peptide fragments (Table 2, peptides 10–14) according to fibrinogen Aα-chain cleavage sites for fibrolase and for Cbfib enzymes and these fragments were used as potential substrates for fibrinogenolytic metalloproteases ($Lys^{413}$-$Leu^{414}$, $Pro^{516}$-$Met^{517}$). Lebetase cleaved the peptide EYHTEKLVTS at position $Lys^6$-$Leu^7$ and the peptide FFSPMLGE at position $Pro^4$-$Met^5$. On the basis of cleavage of peptides (Table 2, peptides 10, 11, 12, 13, 14) we can conclude that lebetase hydrolyzes Aα-chain of fibrinogen at positions $Lys^{413}$–$Leu^{414}$, $Pro^{516}$–$Met^{517}$. The same positions are attacked also by VLAIP but the reaction is considerably slower (Table 3). Recently the cleavage of the synthetic octapeptide (Abz-His-Thr-Glu-Lys-Leu-Val-Thr-Ser-Dnp) containing the fibrolase scissile $Lys^{413}$-$Leu^{414}$ bond in the α-chain sequence of fibrin has been utilized as a more sensitive and rapid method for evaluation of fibrinolytic activity. This assay mimics conditions in which the desired target

sequence is presented to fibrolase and allows the direct quantitative evaluation of biological activity (Swenson and Markland 2005). In an effort to address question about the specificity of the cleavage site, and the requirements for efficient cleavage, synthetic peptides representing the sequence surrounding the $Lys^{413}$-$Leu^{414}$ bond in the Aα chain of fibrinogen were created. These peptides were synthesized with various substitutions for both $Lys^{413}$ and $Leu^{414}$. The data from these experiments indicate that alteration at the Lys position in the octapeptide has a minimal effect on activity, since fibrolase exhibits similar cleavage kinetics for all the peptides with substitutions at the lysine position. The notable exceptions being $Leu^{413}$, with approximately 10-fold lower turnover rate, and $Asn^{413}$, with 30-fold greater turnover rate. These results suggest that fibrolase-mediated cleavage is directed to an X-Leu bond. In contrast, with single amino acid substitutions for the leucine residue ($Leu^{414}$), only the phenylalanine-substituted peptide serves as a fibrolase substrate. This observation is consistent with the common hydrophobic characteristics shared by phenylalanine and leucine. These results suggest that the fibrolase cleavage specificity may be directed toward X-Hyd, where Hyd is a bulky hydrophobic residue (Swenson and Markland 2005).

Lebetase cleavage sites in the "bait" region of $\alpha_2$-macroglobulin ($\alpha_2$-M) and PZP were determined by Edman degradation analysis of the digestion products (Saidi et al 1999). Corresponding 6-10 amino acid residues containing peptide fragments which reproduce the sequence around the cleavage site of the "bait" region of $\alpha_2$-M (Table 2, peptides 7–9, 17–19) and the sequence around the cleavage site of PZP (Table 2, peptides 5, 6, 15) were used as substrates for lebetase and VLAIP. Both enzymes cleave the same bonds in these peptides (Table 3). $\alpha_2$-M is primarily cleaved in the "bait" region by lebetase at the $Arg^{696}$–$Leu^{697}$ bond, which is one of the most common cleavage sites in $\alpha_2$-M by proteinases. The other two cleavage sites in $\alpha_2$M by lebetase are $Gly^{679}$–$Leu^{680}$ and $His^{694}$–$Ala^{695}$. In PZP, the only cleavage site was $Pro^{689}$–$Gln^{690}$ (Saidi et al 1999) as determined by Edman degradation analysis of the digestion products.

It has been shown that *V. lebetina* venom contains bradykinin-releasing serine enzymes (Siigur et al 1982). MALDI TOF MS was very effective for detection of bradykinin that was liberated from human plasma after treating with bradykinin-releasing enzyme from *V. lebetina* venom. This method successfully replaces the rat uterus test formely used for kinin detection. Bradykinin is a naturally occurring peptide that plays a role in maintenance of blood pressure. Lebetase was found to remove a C-terminal dipeptide from bradykinin and kallidin at the position $Pro^{7}$-$Phe^{8}$, as well as the other metalloprotease VLAIP does (Table 3). $Pro^{7}$–$Phe^{8}$ bond cleavage is characteristic also for fibrinolytic enzyme fibrolase (Swenson and Markland 2005) and several other metalloproteinases, such as rabbit liver metalloendopeptidase (Kojima et al 1995) and endothelin-converting enzyme-1 (ECE-1) (Johnson et al 1999). Cleavage of bradykinin and kallidin (Lys-bradykinin) by lebetase (an important side effect of the enzyme) destroys their biological (vasodilatory) activity. Viperidae venoms contain several serine proteases that release bradykinin (Siigur et al 1982) and metalloproteases that cleave bradykinin, therefore the detection of bradykinin-releasing enzymes without metalloprotease inhibitors in crude venom is inaccurate.

Glucagon was relatively poor substrate for lebetase; glucagon was only slightly hydrolyzed at position $Tyr^{10}$–$Ser^{11}$ (Table 2). However, hemorrhagic metalloproteinase toxin b from *Crotalus atrox* venom hydrolyzed glucagon, producing six peptide fragments (Hagihara et al 1995). Lebetase has much narrower specificity, hydrolyzing only one bond in glucagon.

Comparison of amino acid sequences for a variety of substrates shows that lebetase has no strict P1-P1′ specificity requirement. Lebetase cleaves insulin B-chain at $Ala^{14}$–$Leu^{15}$, $Tyr^{16}$–$Leu^{17}$, Pro–Gln site in different peptides and proteins. Lebetase hydrolysis at sites with a Pro residue at P1 was observed with bradykinin, kallidin, substance P, peptide fragments of PZP and fibrinogen Aα-chain as substrates. It is not clear from this study whether Pro is preferred at this position as sites containing Ala, Tyr, Gly, Lys and Arg residues at P1 position were also cleaved by lebetase. In this respect, lebetase is similar to some other metalloproteinases, which also tolerate a variety of residues in the P1 position (Johnson et al 1999).

We have evaluated the activity of lebetase against a number of peptide substrates and conclude that lebetase exhibits both size and sequence specificity in the hydrolysis of peptides. As with other proteinases, recognition of the cleavage site involves discrete amino acid binding subsites on either side of the scissile bond.

It is noteworthy that substance P and an eight-residue peptide fragment of PZP are good substrates for lebetase. In both peptides lebetase cleaves Pro–Gln bond. To our knowledge, there are no data about substance P cleavage by other snake venom proteinases. Several other metalloproteinases from different sources cleave substance P at different sites (Johnson et al 1999, Teahan et al 1989, Niedwiecki et al 1992), but no cleavage was observed at Pro-Gln site.

VLFXA hydrolyzed 6–9 amino acid residues containing peptide fragments synthesized according to the physiological cleavage region of human factor X and human factor IX. VLFXA cleaved the Arg-Ile bond in peptides 1–3 and the Arg-Val bond in peptide 4 (Table 4). VLFXA cleaved more effectively nine amino acid residues containing factor X fragment than nine amino acid containing factor IX fragment. The factor IX fragment was not entirely cleaved after 20 h treatment with VLFXA. MALDI-TOF MS was highly efficient for the recovery and identification of peptides released by VLFXA hydrolysis. We used these peptides for localization of VLFXA in different steps of purification as well as in inhibitory tests.

Table 4. MALDI-TOF analysis of reaction mixture after hydrolysis of substrates by factor X activator from *V. lebetina* venom

| Number | Substrate P1↓P1´ | $(M_w+H)^+$ | | Fragments | $(M_w+H)^+$ | |
|---|---|---|---|---|---|---|
| | | Calc. | MALDI | | Calc. | MALDI |
| 1 | TR↓IVGG | 601.69 | 601.89 | TR<br>IVGG | 275.29<br>344.39 | 276.18<br>345.09 |
| 2 | LTR↓IVGG | 714.85 | 715.44 | LTR<br>IVGG | 388.45<br>344.39 | 389.29<br>345.10 |
| 3 | NNLTR↓IVGG | 943.05 | 944.48 | NNLTR<br>IVVG | 616.66<br>344.39 | 617.70<br>345.10 |
| 4 | NDFTR↓VVGG | 964.03 | 964.47 | NGFTR<br>VVGG | 651.66<br>330.37 | 651.67<br>331.18 |

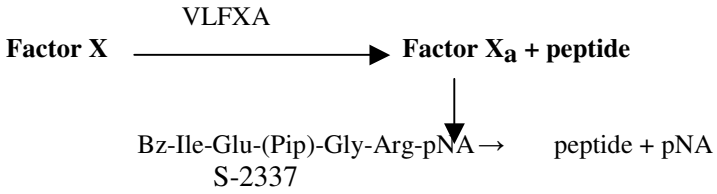
↓ indicates the cleavage site of peptide by factor X activator

### 3.3.2. Cleavage of proteins

#### 3.3.2.1. Activation of factor X (II)

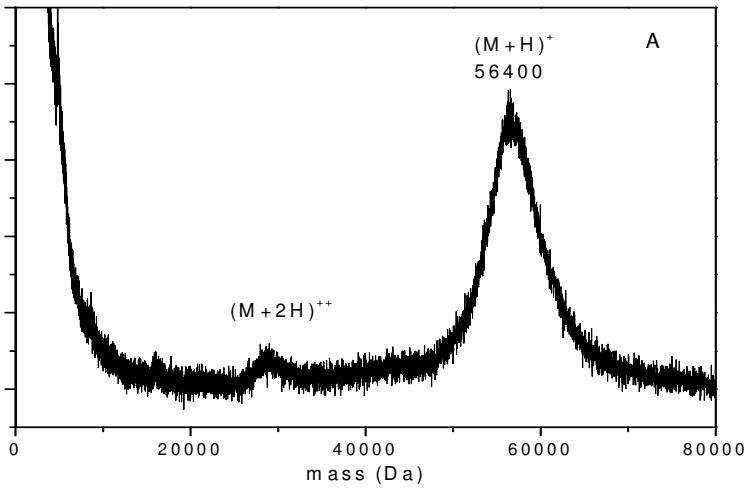
The purified factor X activator (VLFXA) from *V. lebetina* venom had no effect on fibrinogen, prothrombin and plasminogen, indicating that the activation of factor X was specific. The effects of VLFXA on human factor X were studied by measuring the amidolytic and the coagulant activities of the activated factor X (factor $X_a$). The activators convert the inactive factor X in the presence of $Ca^{2+}$ ions to the active form $X_a$ which activity was detected using substrate S-2222 in a complex two-stage reaction. The activation without $Ca^{2+}$ was about 5%. The enzyme has a maximum activity on factor X in the interval of pH 7.5–8.0. The factor X activating enzymes themselves have no amidolytic activity against factor $X_a$ substrate S-2222 or S-2337, while *V. lebetina* venom itself hydrolyzes this substrate.

**Detection of factor X**:

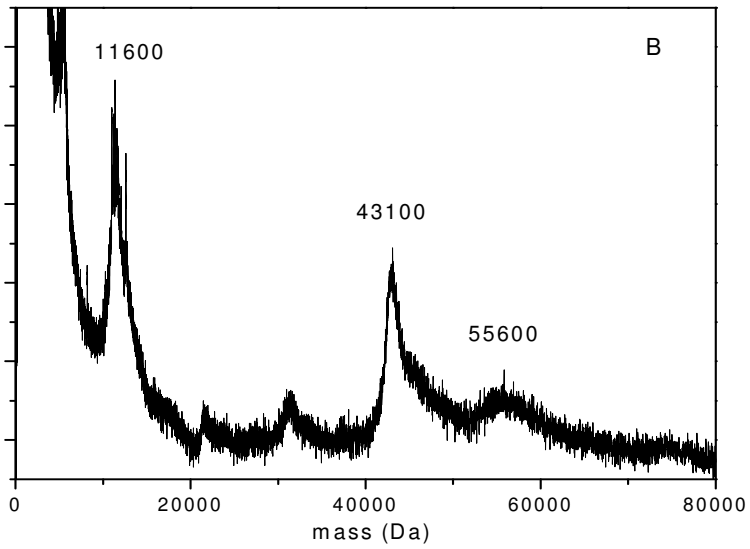



Morita (1998) proposed that RVV-X primarily recognizes the calcium-bound conformation of the Gla domain in factor X via the regulatory subunit (LC1 + LC2), and the subsequent conversion of factor X to active factor $X_a$ is catalyzed by the catalytic subunit. It seems that the catalytic mechanism of VLFXA should be similar to that of RVV-X.

The catalytic cleavage of human factor X (Lot 27H9305 Sigma) by the activators from *V. lebetina* (VLFXA) and *V. russelli* (RVVXAE, Sigma) venoms was examined by MALDI-TOF MS. The masses of cleavage products of human factor X were 43.1 and 11.6 kDa after treating with VLFXA (Fig. 12) and RVV-X. The activation was already detectable after 5 min hydrolysis in the case of VLFXA. On the basis of cleavage of peptides (Table 4) and human factor X we can conclude that VLFXA hydrolyzes human factor X at positions $Arg^{52}$-$Ile^{53}$ and human factor IX precursor at positions $Arg^{226}$-$Val^{227}$. Factor X can be completely converted to factor $X_a$ by VLFXA and the specific activity of factor $X_a$ is the same as that of factor $X_a$ obtained after activation by the activator from Russell's viper venom.




A




B

Fig. 12. (A) MALDI-TOF mass spectrum of human factor X; (B) MALDI-TOF mass spectrum of cleavage products of human factor X by VLFXA.

Calcium ions are not necessary for the cleavage of peptides by VLFXA. *V. russelli* factor X activator cleaves apoprotein AI from human high-density lipoprotein without calcium ions (Amphlett et al 1982). Our observation supports the argument that calcium ions interact with substrate, rather than with the enzyme.

Hydrolytic activity of VLFXA against peptide substrates was inhibited by EDTA (20 mM) and *o*-phenanthroline (5 mM), 2 h, pH 8.0, at 37 °C. PMSF (1 mM, 2 h, pH

7.2, 37 °C) has no effect on the activity. Peptide cleavage was determined by MALDI-TOF MS. These results show that the enzyme is a metalloproteinase.

Judged from the degradation products of human factor X, the cleavage specificity of factor X for VLFXA is the same as that for RVV-X. The cleavage of peptide fragments of factor X and factor IX confirms this suggestion. The molecular structure of VLFXA appears to resemble that of the factor X activator from *V. russelli* venom.

### 3.3.2.2. Cleavage of proteins by VLAIP (VI)

Substrate specificity of the enzyme was studied against different proteins. The enzyme hydrolyses azocasein at pH 8.3 with the specific activity of 3700 U/mg (1 unit is the amount of enzyme which caused the increase in absorbancy at 390 nm of 0.001 per 2 h) and has weak gelatinolytic activity. EDTA or 1,10-phenanthroline (10 mM, preincubation for 1 h) inhibited the hydrolytic activity of the enzyme. No inhibition occurred with PMSF (1 mM, preincubation for 1 h). These experiments confirm the metalloproteinase nature of the enzyme.

Cleavage of fibrinogen is shown in Fig. 13. The enzyme (50 µg/ml) digested Aα-chain completely within 0.5 h. The Bβ-chain was cleaved more slowly and the γ-chain was left intact even after 22 h incubation.

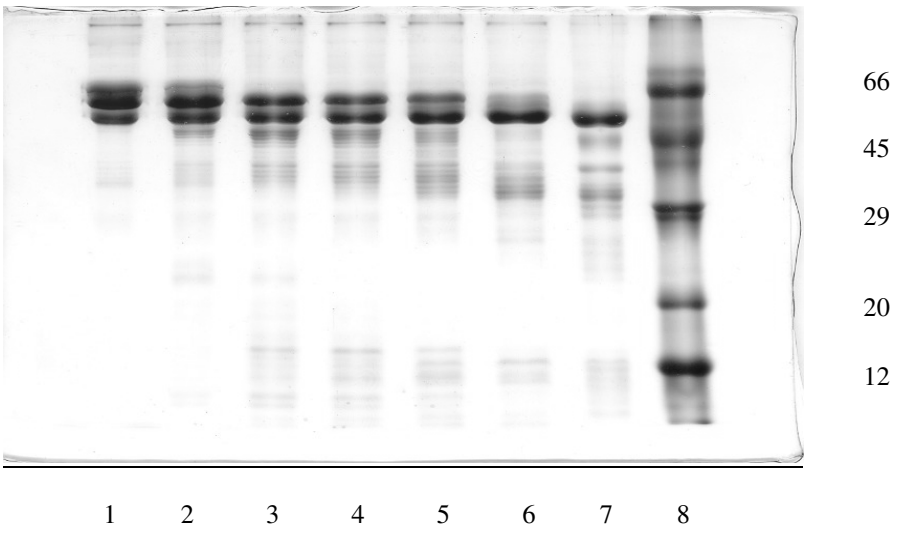



Fig. 13. Reduced SDS electrophoresis pattern of fibrinogen degradation products by VLAIP (12.5% gel). Lanes 1-7 fibrinogen incubated with VLAIP for 0, 5, 30 min, 1, 2, 6, and 22h. Lane 8, molecular mass standards

The enzyme did not hydrolyse fibrin in 1% fibrin plate. Homodimeric VAP-1 (Masuda et al 2000) and monomeric halysase (You et al 2003) also completely degrade the fibrinogen Aα-chain. Collagen I, collagen IV and fibronectin were not cleaved after 6 h treatment with VLAIP. Weak cleavage of fibronectin and collagen I was detectable after 22 h treatment (data not shown).

### 3.3.3. Cellular specificity (VI)

#### 3.3.3.1. Effect of VLAIP on HUVEC adhesion

The primary effects of VLAIP on HUVECs were induction of changes in the cell shape and in the attachment of cells to the substrate followed by their detachment, subsequent aggregation and cell death. After 1 h treatment of cells with 20 μg/ml of VLAIP pronounced changes were observed, whereas the effect of fraction II from CM-52 containing VLAIP as a minor component was considerably less expressed. The most active was the venom itself requiring lesser concentrations to produce similar rapid effects on HUVECs. However, as the crude venom contains also other components that may affect HUVECs, its effect is a combination of effects of different components including metalloproteases, disintegrins, C-type lectins, L-amino acid oxidase etc.

Effect of VLAIP on HUVEC adhesion to immobilized ECM proteins fibronectin, collagen I, collagen IV, vitronectin and fibrinogen was studied in more detail. VLAIP inhibited significantly adhesion of endothelial cells from 3-d passage on all substrates in a concentration of 1 μg/ml, the effect was even more pronounced in a concentration of 10 μg/ml. At VLAIP concentration of 1 μg/ml the average number of adhered cells was about 20% of control on fibrinogen and between 40-50% on vitronectin and collagen IV. The effect of 1 μg/ml of VLAIP was less so obvious on attachment on fibronectin and collagen I, the average number of adhered cells being around 70-80% of untreated controls. 10 μg/ml of VLAIP inhibited the adhesion of at least 80% of cells on all substrates and on fibrinogen-coated wells no adhered cells were visually seen. We also used laminin I for cell culture substrate, but no adhesion of HUVECs occurred on laminin even without VLAIP treatment (data not shown). In the experiments with cells from 7-th passage for adhesion experiments, VLAIP decreased cell adhesion only in concentrations as high as 10 μg/ml, except for collagen IV coating where the effect could be seen already with 1 μg/ml VLAIP, and fibrinogen coating, where the inhibiting effect was significant even in the concentration of 0.1 μg/ml.

Multiple integrins are expressed on HUVEC cells, allowing adhesion to extracellular matrix proteins. The disintegrin-like domains of reprolysins may interact with integrins to mediate cell-cell adhesion (e.g. HUVECs) or the attachment of cells to extracellular matrix. It has been shown that different RGD-containing disintegrins (e.g. echistatin, kistrin, flavoridin) inhibit HUVECs adhesion to fibronectin and vitronectin with variable activity. This interaction with HUVECs appears to be selectively mediated by $\alpha_v\beta_3$ receptors (Juliano et al 1996). Homodimeric RGD-containing disintegrin contortrostatin effectively blocked adhesion of HUVECs to immobilized vitronectin. Detachment of HUVECs from vitronectin by contortrostatin induced apoptosis (Zhou et al 1999). ECD is one of the most common alternative motifs in non-RGD-containing snake venom disintegrins. The ECD-containing disintegrin-like domain of the sperm surface protein, fertilinβ (ADAM-2), is thought to bind to an $\alpha_6\beta_1$ integrin receptor on the egg oolemma (Almeida et al 1995). Halysase which contains DECD sequence in its disintegrin domain more strongly inhibits the endothelial cell adhesion to fibronectin that binds to integrin $\alpha_5\beta_1$ on the cell surface than to other ECM proteins (You et al 2003). Our results showed that VLAIP inhibited most strongly HUVECs adhesion to fibrinogen, but the inhibiting effects of VLAIP on HUVECs adhesion to other ECM proteins were also significant. Wu et al. (2001) demonstrated that graminelysin I that contains only a metalloproteinase domain inhibits the adhesion

of HUVECs to immobilized fibrinogen in a concentration–dependent manner. The exact mechanism by which VLAIP exerts its effects remains to be established.

### 3.3.3.2. Induction of endothelial cell apoptosis by VLAIP

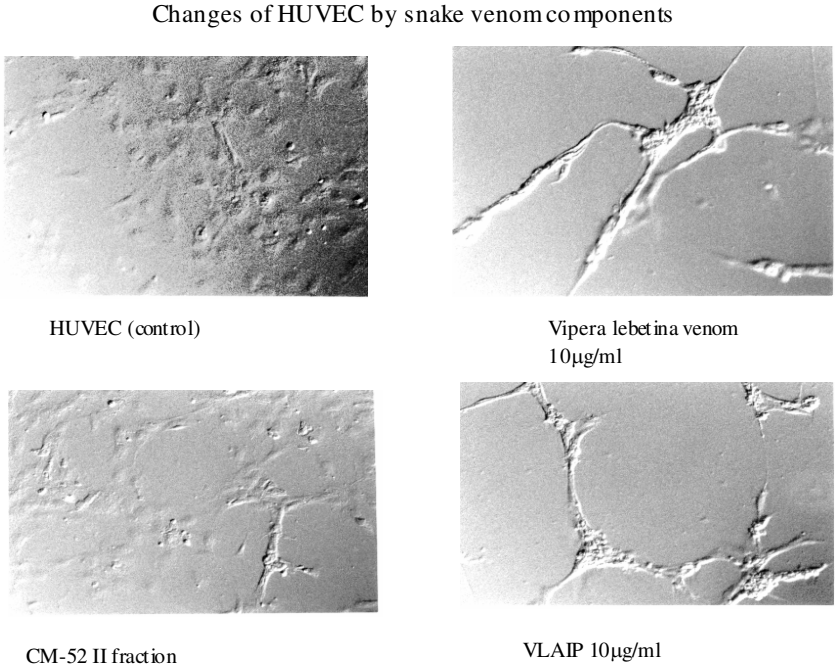



Fig. 14. Changes of HUVECs by snake venom components.

To establish if the cell death occurred due to apoptosis we analysed DNA fragmentation, a marker of apoptosis, using electrophoretic analysis of DNA isolated from VLAIP-treated HUVECs. As shown in Fig. 15, characteristic DNA ladder was observed after 24 h treatment of HUVECs with VLAIP (lanes 3 and 4).

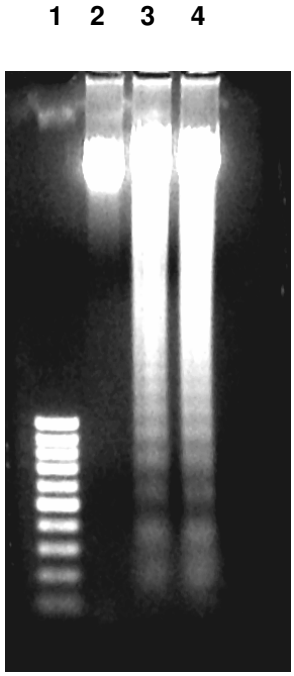



Fig. 15. Electrophoretic patterns of DNA fragmentation in VLAIP-treated HUVECs. *Lane 1*: 100 bp DNA ladder marker (Fermentas); *Lane 2*: HUVECs cultured in medium with 10% FCS for 24 h, treated with PBS; *Lane 3*: HUVECs cultured in medium with 10% FCS for 24 h, treated with VLAIP (10 µg/ml); *Lane 4*: HUVECs cultured in medium with 10% FCS for 24 h, treated with VLAIP (20 µg/ml). The total cellular DNA was isolated and subjected to electrophoretic separation on a 1.5% agarose gel and visualized wih ethidium bromide staining.

The induction of apoptosis was confirmed by monitoring phosphatidylserine externalization using annexin V-FITC staining and flow cytometric analysis. The apoptosis inducing capability is stronger than in case of graminelysin I (Wu et al 2001) and seems to be comparable to metalloproteases that contain metalloprotease and disintegrin part both (Wang et al 2003, You et al 2003). It has been shown that both metalloprotease activity and the disintegrin-like domain of halysase contribute to the induction of HUVEC apoptosis (You et al 2003).

The effects of VLAIP are cell type specific since 48 h incubation of HeLa and NIH3T3 cells with 20 µg/ml of VLAIP revealed only slight changes in the attachment of cells to the substrate (data not shown).

Our results demonstrate that VLAIP from *Vipera lebetina* snake venom is a potent promoter of apoptosis in vascular endothelial cells. Further studies are needed for identification of the molecular mechanism by which VLAIP induces apoptosis in HUVEC. VLAIP might be used as a tool for studying the functional mechanism of vascular endothelial cells.

## CONCLUSIONS

The main results of the study are following:

1. An apoptosis-inducing metalloproteinase (VLAIP) has been isolated from *Vipera lebetina* venom. The enzyme is a heterodimeric glycoprotein with molecular mass of about 106 kDa. It is the first heterodimeric metalloproteinase found in snake venoms.

2. VLAIP hydrolyzes azocasein, fibrinogen and oxidized insulin B-chain. It induces apoptosis in HUVEC cells confirmed by monitoring phosphatidylserine externalization, flow cytometric analysis and characteristic DNA ladder. VLAIP inhibits endothelial cell adhesion to extracellular matrix proteins: fibrinogen, fibronectin, vitronectin, collagen I, collagen IV but not to laminin.

3. Proceeding from the conserved intrachain disulfide bond pattern of 6-Cys metalloproteases, the S-S bridges in VLAIP chains should look as follows: $Cys^{314}$-$Cys^{394}$, $Cys^{354}$-$Cys^{378}$ and $Cys^{356}$–$Cys^{361}$. $Cys^{369}$ remains free and could be used for formation of interchain disulfide bond between the two monomers. $Cys^{369}$ seems to be typical for vascular apoptosis inducing proteases, many other snake venom metalloproteases have different amino acids in this position

4. The substrate specificity of VLAIP and lebetase (a fibrinolytic enzyme from *V. lebetina* venom) has been studied with 6-9 amino acid residues containing peptides. Both VLAIP and lebetase have kininase activity hydrolyzing $Pro^{7}$-$Phe^{8}$ bond in bradykinin. Cleavage of bradykinin and kallidin (Lys-bradykinin) by lebetase (an important side effect of the enzyme) destroys their biological (vasodilatory) activity. The cleavage site of substance P is different by VLAIP and lebetase. They both hydrolyse oxidised insulin B-chain. VLAIP cleaves oxidized insulin B-chain only in one position $Ala^{14}$-$Leu^{15}$. The best peptide substrate for lebetase was substance P.

5. A factor X activating proteinase (VLFXA) has been isolated and characterized. It is the first fully sequenced P-IV type snake venom metalloproteinase. The enzyme comprises three chains: one heavy chain (57.5 kDa) and two light chains (17.4 and 14.5 kDa). The amino acid sequences deduced from the cDNAs encoding the chains of VLFXA have been proven by the masses of the tryptic peptides. The sites of cleavage of human factor X and factor IX are determined by MALDI TOF MS and correspond to the physiological cleavage sites of both factors.

6. VLFXA is a useful diagnostic agent for detecting of factor X in human blood.

7. VLAIP might be used as a tool for studying apoptosis processes and for designing potential drugs.

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**ARTICLE I**

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**ARTICLE II**

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**ARTICLE III**

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**ARTICLE IV**

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Table 1
Molecular masses of *V. lebetina* and *V. berus berus* proteases

| Enzyme | Nonreduced Da | Reduced Da |
|---|---|---|
| Lebetase | 22912 [22]* | 23700 [18] |
| VLAIP | 106000* | 60000 |
| VLFXA | 86500–91000 [20]* | 57500; 17400; 14500 [20] |
| VLFVA | 28400 [17]* | 30000 [17] |
| $\beta$-fibrinogenase | 42200 [14]* | 52500 |
| $\alpha$-fibrinogenase | 31100 [14]* | 37000 |
| VBFXAEI | 97500 | 56900; 16800; 12000 |
| VBFXAEII | 125900 | 59300 |
| VBFXAE | 38000 [13] | 38000 [13] |

*Detected by MALDI-TOF MS.

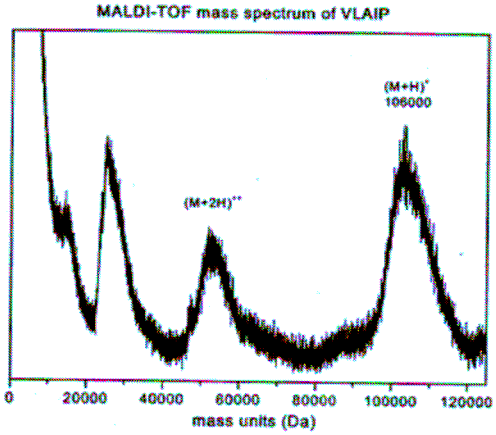


Fig. 1. MALDI-TOF mass spectrum of VLAIP. $(M + H)^+$ was indicated as the molecular ion while $(M + 2H)^{++}$ as double charged ions. Matrix was ferulic acid.

slowly B$\beta$-chain. *V. lebetina* venom $\alpha$-fibrinogenase degrades only A$\alpha$-chain [14]. None of them attacks $\gamma$-chain of fibrinogen. *V. lebetina* venom contains thermostable $\beta$-fibrinogenase that preferably cleaves $\beta$-chain of fibrinogen [14]. We have shown that cleavage patterns of fibrinogen treated with venom proteases are different [14,18]. We synthesized 6–10 amino acid containing peptide fragments according to fibrinogen A$\alpha$-chain cleavage site regions for various venom metalloproteases ($Lys^{413}$–$Leu^{414}$, $Pro^{516}$–$Met^{517}$) [26]. These peptides were used as potential substrates for fibrinogenolytic metalloproteases. Lebetase cleaved the peptide EYHTEKLVTS at position $Lys^6$–$Leu^7$ and the peptide FFSPMLGE at position $Pro^4$–$Met^5$ [26], the same positions are attacked also by VLAIP but the reaction is considerably slower (Table 2). VBFXAEI cleaved $Lys^6$–$Leu^7$ bond and more slowly $Tyr^2$–$His^3$ bond in EYHTEKLVTS, VBFXAEII catalyzed only the cleavage of $Lys^6$–$Leu^7$ bond (Table 3). Neither *V. berus berus* enzyme cleaved the peptide FFSPMLGE.

The purified factor X activator (VLFXA) from *V. lebetina* venom had no effect on fibrinogen, prothrombin, plasminogen, indicating that the activation of factor X was specific [20]. The effects of VLFXA, VBFXAE, VBFXAEI and VBFXAEII on human factor X were studied by measuring the ami-

**ARTICLE V**

Reprinted from Biochim. Biophys. Acta 1702, Siigur E., Aaspõllu A., Trummal K., Tõnismägi K., Tammiste I., Kalkkinen N., Siigur J. Faxtor X activator from *Vipera lebetina* venom is synthesized from different genes, pp. 41-51. Copyright (2004), with permission from Elsevier.

**ARTICLE VI**

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**ARTICLE VII**

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**ABBREVIATIONS**

| | | |
|---|---|---|
| ACN | - | acetonitrile |
| ADAM | - | A Disintegrin And Metalloproteinase |
| ADAMTS | - | A Disintegrin And Metalloproteinase with Thrombospondin Motifs |
| CM | - | carboxymethyl |
| Cys-CAM | - | Cys-carboxamidomethyl |
| DCC | - | *N*-dicyclohexylcarbodiimide |
| DCM | - | dichloromethane |
| DHB | - | dihydroxybenzoic acid |
| DIEA | - | diisopropylethylamine |
| DMSO | - | dimethyl sulfoxide |
| HC | - | heavy chain |
| HPLC | - | high performance liquid chromatography |
| MALDI-TOF MS | - | matrix assisted laser desorption ionization time of flight mass spectrometry |
| ECM | - | extracellular matrix |
| EDTA | - | ethylenediaminetetraacetic acid |
| FXA | - | factor X activator |
| HOBT | - | 1-hydroxybenzotriazole |
| HUVEC | - | human umblicial vein endothelial cells |
| LC | - | light chain |
| LC-MS/MS | - | liquid chromatography-electrospray ionization time of flight mass spectrometry |
| MMP | - | matrix metalloproteinase |
| MTT | - | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium |
| NMP | - | *N*-methylpyrrolidone |
| PAA | - | polyacrylamide |
| PMSF | - | phenylmethylsulfonyl fluoride |
| PZP | - | pregnancy zone protein |
| SDS-PAGE | - | sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis |
| SVMP | - | snake venom metalloprotease |
| TACE | - | TNF-alpha converting enzyme |
| TFA | - | trifluoroacetic acid |
| TFMSA | - | trifluoromethane sulfonic acid |
| TGF | - | transforming growth factor |
| TNF | - | tumor necrosis factor |
| VEGF | - | vascular endothelial growth factor |
| VLAIP | - | *Vipera lebetina* endothelial cell apoptosis inducing metalloproteinase |
| VLFXA | - | *Vipera lebetina* factor X activator |

**ABSTRACT**

Snake venoms are rich sources of biologically active proteins, metalloproteinases representing the vast majority of the venom components. Three metalloproteinases from *Vipera Lebetina* venom, that have biomedical importance, have been characterized and their substrate specificity against proteins and peptides has been studied. Two of them - factor X activator (VLFXA) and endothelial cell apoptosis inducing proteinase (VLAIP) have been purified during this work.

A heterodimeric glycoprotein (VLAIP) that induces apoptosis in endothelial cells is purified to homogeneity. This metalloproteinase has strict specificity on oxidized insulin B chain cleaving Ala14-Leu15 bond only but in substance P three bonds (Gln5-Gln6; Phe7-Phe8; Gly9-Leu10) are hydrolysed. The cleavage sites (as detected by MALDI-TOF MS) in fibrinogen fragments 406-417 and 513-520, $\alpha_2$-macroglobulin fragments 693-700 and 676-684, and PZP fragment 686-693 are identical with cleavage sites of lebetase, a fibrinolytic enzyme from V. lebetina venom. Both enzymes exhibit kininase activity, hydrolysing Pro7-Phe8 bond in bradykinin.

The induction of apoptosis by VLAIP in HUVEC cells has been shown by phosphatidylserine externalization, flow cytometric analysis and a typical apoptotic DNA ladder. VLAIP induces changes in attachment of HUVEC cells to the substrate and causes cell death. We demonstrated also that VLAIP inhibits endothelial cell adhesion to extracellular matrix proteins: fibrinogen, fibronectin, vitronectin, collagen I and collagen IV but not to laminin.

A procoagulant factor X activating metalloproteinase (VLFXA) was isolated and characterized. It is a three-chain glycoprotein comprising a heavy chain (57.5 kDa) and two light chains (17.4 and 14.5 kDa). The light chain LC2 is not glycosylated. VLFXA has multiple molecular forms in the pH interval of 5.3-5.9. The masses of tryptic peptides determined by MALDI-TOF MS confirmed the primary structures of all three chains deduced from the cDNA sequences of the proteins. VLFXA activates factor X via cleavage of the Arg52-Ile53 bond in human factor X and factor IX by cleavage of the Arg226-Val227 bond in human factor IX (very slowly). It does not activate prothrombin nor has any effect on fibrinogen.

## KOKKUVÕTE

Maomürkides on suurel hulgal bioloogiliselt aktiivseid valke, millest metalloproteinaasid moodustavad olulise osa. *Vipera lebetina* mürgist eraldati kolm biomeditsiiniliselt olulist metalloproteinaasi, iseloomustati ja uuriti nende spetsiifilisust erinevate valkude ja peptiidide suhtes. Kaks neist – faktor X aktivaator (VLFXA) ja endoteelirakkude apoptoosi indutseeriv proteinaas (VLAIP) – on puhastatud käesolevas töös.

Heterodimeerne glükosüleeritud endoteelirakkude apoptoosi indutseeriv metalloproteinaas (VLAIP) on rangelt spetsiifiline oksüdeeritud insuliini B-ahela suhtes, hüdrolüüsides ainult $Ala^{14}$-$Leu^{15}$ sidet, substance P-s aga kolme sidet ($Gln^{5}$-$Gln^{6}$, $Phe^{7}$-$Phe^{8}$, $Gly^{9}$-$Leu^{10}$). Lõhkumissaidid (detekteerituna MALDI-TOF MS abil) fibrinogeeni fragmentides 406-417 ja 513-520, $\alpha_2$-makroglobuliini fragmentides 693-700 ja 676-684 ning PZP fragmendis 686-693 on identsed *Vipera lebetina* fibrinolüütilise ensüümi lebetaasi lõhkumissaitidega. Nii VLAIP kui lebetaas omavad kininaasset aktiivsust, hüdrolüüsides bradikiniini $Pro^{7}$-$Phe^{8}$ sidet.

HUVEC rakkude apoptoosi indutseerimine VLAIP-i poolt on näidatud fosfatidüülseriini eksternalisatsiooniga, voolutsütomeetrilise analüüsiga ja tüüpilise DNA fragmentatsioooni redeli abil. VLAIP indutseerib muutusi HUVEC rakkude kinnitumisel substraadiga ja põhjustab apoptootilise rakusurma. Näitasime ka, et VLAIP inhibeerib endoteelirakkude adhesiooni ekstratsellulaarse maatriksi valkudega: fibrinogeeni, fibronektiini, vitronektiini, kollageen I ja kollageen IV-ga, kuid mitte laminiiniga.

Eraldati ja iseloomustati prokoagulantne faktor X aktiveeriv metalloproteinaas (VLFXA). See on kolmeahelaline glükoproteiin, mis koosneb raskest ahelast (57,5 kDa) ja kahest kergest ahelast (17,4 ja 14,5 kDa). Kerge ahel LC2 ei ole glükosüleeritud. VLFXA-l on mitmeid molekulaarseid vorme pH intervallis 5,4 - 5,9. MALDI-TOF MS poolt määratud trüpsiiniga lõhkumisel saadud peptiidide massid kinnitasid valkude cDNA-de järjestustest tuletatud kõigi kolme ahela primaarstruktuure. VLFXA aktiveerib faktor X, lõhkudes inimese faktor X-s $Arg^{52}$-$Ile^{53}$ sidet, ja faktor IX, lõhkudes $Arg^{226}$-$Val^{227}$ sidet (väga aeglaselt). VLFXA ei aktiveeri protrombiini ega hüdrolüüsi fibrinogeeni.

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Orgaaniline ja biokeemia; valkude struktuur ja funktsioon; valkude eraldamise meetodid, peptiidsüntees, MALDI-TOF mass-spektromeetria
Tegevusuunad kajastuvad täpsemalt artiklites

**LIST OF PUBLICATIONS**


1. Trummal K., Vija H., Subbi J., Siigur J. MALDI-TOF mass spectrometry analysis of substrate specificity of lebetase, a direct-acting fibrinolytic metalloproteinase from *Vipera lebetina* snake venom. Biochim. Biophys. Acta, 2000, 1476(2), 331-336.

2. Siigur J., Trummal K., Tõnismägi K., Siigur E., Vija H., Subbi J. MALDI-TOF mass spectrometry analysis of substrate specificity of metalloproteinases from *Vipera lebetina* snake venom. XIII World Congress of the International Society on Toxinology, Paris, Sept 18-22, 2000, Abstracts, p. 123.

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