PROTEINASES FROM *VIPERA LEBETINA* SNAKE VENOM AFFECTING HEMOSTASIS

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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 the candidate:

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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 Siigur, E., **Aaspõllu, A**., Tu, A.T., Siigur, J. (1996) cDNA Cloning and Deduced Amino Acid Sequence of Fibrinolytic Enzyme (Lebetase) from *Vipera lebetina* Snake Venom. Biochem. Biophys. Res. Commun. 224, 229-236.

II Siigur, E., **Aaspõllu, A**., Siigur, J. (1999) Molecular Cloning and Sequence Analysis of a cDNA for Factor V Activating Enzyme, a Coagulant Protein from *Vipera lebetina* Snake Venom. Biochem. Biophys. Res. Commun. 262, 328-332.

III Siigur, E., **Aaspõllu, A**., Siigur, J. (2001) Sequence diversity of *Vipera lebetina* snake venom gland serine proteinase homologs – result of alternative-splicing or genome alteration. Gene 263, 199-203.

IV 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.

V Siigur, E., **Aaspõllu, A**., Siigur, J. (2003) Anticoagulant serine fibrinogenases from *Vipera lebetina* venom: structure-function relationship. Thromb. Haemost. 89, 826-831.

VI 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.

VII 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, 46-61.

VIII **Aaspõllu, A**., Siigur, J. Siigur, E. (2005) cDNA cloning of a novel P-I lebetase isoform Le-4. Toxicon 46, 591-594.

INTRODUCTION

Snakes have been intriguing people over a thousand years with their unusual appearance, snaking as a mode of locomotion, particular behaviour and finally the toxicity of several species. Over centuries much research has been conducted on snakes which systematically belong to the Class *Reptilia*, Order *Squamata*, and Suborder *Ophidia.* Special interest has been focused on snake venoms and envenomation.

By now more than 2000 species of snakes have been described (Fry 2005, Vidal 2002; the species number is 2978 according to EMBL Reptile database as of January 2005). Venomous snakes are classified into *Elapidae*, *Viperidae*, *Crotalidae*, *Hydrophidae* and *Colubridae* families. Snake venoms are secreted and stored in venom glands. These venoms are rich sources of biologically active compounds, which are primarily intended for killing prey and for aiding their digestion. The content of venoms includes organic and mineral components, affecting neurotransmission and the hemostatic system. Neurotoxins are typical of *Elapidae* (mambas, cobras, and particularly the Australian snakes, the most toxic snakes in the world) and *Hydrophidae* (sea snakes) venoms (Mebs 1990, Fry 1999). Hemostatically active components are more general in *Viperidae* and *Crotalidae* (vipers and rattlesnakes) venoms (Markland 1998). Consequently, envenomation by these snakes generally results in persistent bleeding.

Molecular cloning and characterization of enzymatic activity of different proteins of snake venoms helps us understand basic mechanisms of envenomation and blood coagulation as well as develop novel agents for diagnostic and therapeutic purposes. The application field of snake venom components is broad (review Marsh and Williams 2005). The venom components from various snakes have been used for development of routine laboratory tests, like Reptilase™ for titration of fibrinogen-fibrin reaction (Funk et al 1971, Matsuda et al 1985), Protac® for titration of the proteins C and S (Stocker et al 1987, 1988). Several drugs for human treatment have been developed on the basis of snake venom as well. Well-known drugs are Arvin® used in the prevention and treatment of vascular occlusive diseases (Esnouf and Tunnah 1967, Soutar and Ginsberg 1993), Defibrase® used as a defibrinogenating drug (Stocker 1988). Fibrolase, a fibrinolytic metalloproteinase from *Agkistrodon contortrix contortrix,* has proven to be the superior candidate in medical treatment of relevant blood clots in a way other drugs are unable to – through thrombus degradation (Bolger et al 2001). Up to now, several pre-clinical and clinical trials have been carried out using fibrolase (its recombinantly produced, truncated form – alfimeprase) (Ahmed et al 1990, Markland et al 1994, Markland 1996, Toombs 2001b, Swenson and Markland 2005, Ouriel et al 2005). Other pathological conditions, which can be treated with therapeutical agents based on snake venoms, include Alzheimer, inflammation, autoimmune diseases, virus infections, asthma, osteoporosis and cancer (Marcinkiewicz 2005). Beyond clinical applications, snake venom enzymes are useful biochemical tools for molecular studies and for further applications in biotechnology industry (converting intermediates by specific peptide bond cleavage) (Ramos and Selistre-de-Araujo 2006).

The information obtained through the analysis of snake venom components can be additionally used for reconstruction of evolution of snakes and especially snake venoms (Fry and Wüster 2004). Comparison of genomic/proteomic data of snake venoms with that of venomous species from other phyla is expected to open up new horizons, including an understanding of the function and evolution of venomous systems, a clarification of their genetic relationship with the general biology of venomous animals, clues for understanding the diversity and evolution of venom peptides and proteins (Menez et al 2006).

The aim of the present work was molecular cloning and characterization of proteolytic enzymes from the *Vipera lebetina* venom which affect the human hemostatic system for the elucidation of their structure-function relationships. Within the framework of the current work, a set of metalloproteinases and serine proteinase with coagulant and anticoagulant activities having various roles was studied.

The studied metalloproteinases included: lebetase – a direct-acting fibrinolytic enzyme; factor X activator (VLFXA) – an enzyme that converts the coagulation factor X into its activated form; and a novel endothelial cell apoptosis inducing metalloproteinase (VLAIP) – a heterodimeric protein which in addition to apoptotic activity hydrolyzes the Aα-chain and more slowly the Bβ-chain of fibrinogen but does not cleave fibrin.

Three serine proteinases were analyzed: factor V activator (VLFVA) – a coagulant enzyme which participates in the activation of coagulation factor V; and two anticoagulant enzymes: α-fibrinogenase (VLAF) and β-fibrinogenase (VLBF) which degrade the Aα-chain and Bβ-chain of fibrinogen, respectively, without fibrinolysis.

1. REVIEW OF THE LITERATURE

1.1. BRIEF DESCRIPTION OF THE HEMOSTATIC SYSTEM

Hemostasis involves processes intended for maintaining hemodynamic properties of the blood constant.

The blood coagulation cascade is believed to have evolved over 450 million years ago (Davidson et al 2003). In mammals blood coagulation is initiated and regulated by a complex network of interactions regulated by positive and negative feedback loops (Davidson et al 2003). In normal physiological conditions blood coagulation is initiated upon vascular injury. According to Davie et al (1991) initially platelets are activated and they adhere to the site of injury. The platelets then aggregate and form a platelet plug. The activation of platelets also releases compounds that are involved in triggering the coagulation cascade and fibrin formation, which ends in insoluble fibrin clot generation that strengthens the platelet plug (Fig. 1).

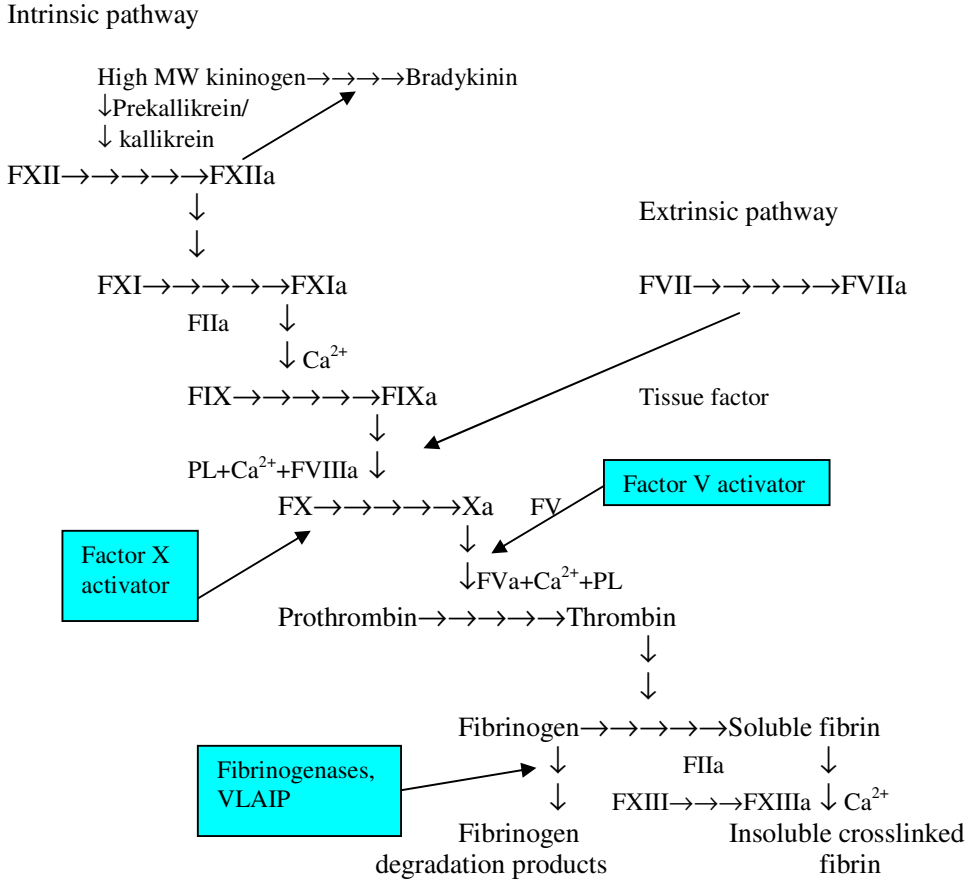

Figure 1. The simplified scheme of blood coagulation and fibrinogenolysis. Steps affected by venomous enzymes of the current work are pointed out.

Fibrinogen molecule consists of two sets of Aα-, Bβ, and γ-chains linked to each other by disulfide bonds (Blombäck 1996). The release of fibrinopeptide A from the N-terminal part of the Aα-chain and fibrinopeptide B from the N-terminal part of the Bβ-chain by thrombin leads to fibrin monomer formation. Produced fibrin monomers become linked by hydrogen bonds into soluble fibrin. In the final step of insoluble fibrin formation the fibrin monomers are covalently cross-linked by activated factor XIIIa.

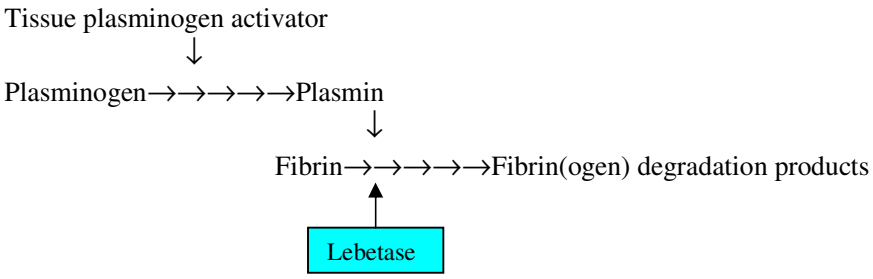

Figure 2. The simplified scheme of fibrinolysis. The step affected by venomous enzymes of the current work is pointed out.

After the fibrin formation the fibrinolysis is initiated and the clot will be lysed within hours. The lytic reaction is catalyzed by plasmin, which is generated from plasminogen (Fig. 2).

1.2. SNAKE VENOM COMPONENTS AFFECTING HEMOSTASIS

Snake venom glands are highly specialized tissues that possess a high capacity of protein production, storage and secretion. The cellular mechanisms of regulation of protein production/secretion processes in this organ are essentially unknown. The venom production was first known to be dependent on the level of proteins stocked in the glandular lamina (Junqueira-de-Azevedo and Ho 2002). Besides proteins (more than 90% of the dry weight), snake venoms contain organic compounds with low molecular mass and inorganic compounds (Bieber 1979).

Numbers of venom proteins and peptides affecting hemostasis have been extensively investigated and characterized (reviewed by Stocker 1990b, Markland 1998c, Braud et al 2000, Matsui et al 2000, Fox and Serrano 2005, Serrano et al 2005, Swenson and Markland 2005).

Many of these proteins are enzymes, eg nucleotidases, phosphodiesterases, L-amino acid oxidases, phospholipases A_2 (PLA_2), metalloproteinases and serine proteinases, whereas others, such as disintegrins and C-type lectins, exhibit no enzymatic activity (Stocker 1990a, Braud et al 2000, Lu et al 2005). Among proteins found in snake venoms, multidomain proteins are represented, containing a catalytic domain and one or several non-catalytic domains. Metalloproteinases are examples of these kinds of proteins, in which catalytic domain either is or is not associated with noncatalytic domain(s) like a disintegrin-like and a cysteine-rich domain and a C-type-lectin-like domain (Bjarnason and Fox 1994, 1995, Fox and Serrano 2005). These noncatalytic domains are proposed to play a major role in the targeting of the proteinase to key proteins with critical functions in hemostasis (Braud et al 2000).

On the other hand, venom proteins can be classified on the basis of their hemostatic action. The following groups can be formed: (I) enzymes that clot fibrinogen; (II) enzymes that degrade fibrin(ogen); (III) plasminogen activators; (IV) prothrombin activators; (V) factor V activators; (VI) factor X activators; (VII) components exhibiting anticoagulant activity including inhibitors of prothrombinase complex formation, inhibitors of thrombin, phospholipases, and protein C activators; (VIII) enzymes with hemorrhagic activity; (IX) enzymes that degrade plasma serine proteinase inhibitors; (X) platelet aggregation inducers including direct-acting enzymes, direct-acting non-enzymatic components, and agents that require a cofactor; (XI) platelet aggregation inhibitors including: α-fibrinogenases, 5'-nucleotidases, phospholipases, and disintegrins (Markland 1998c).

It was estimated earlier that a venom gland synthesizes 50-60 proteins (Matsui et al 2000), by now the number of proteins detected in snake venoms is much greater (Bazaa et al 2005, Li et al 2004, Serrano et al 2005). Venom proteomics has still been lagging in the sense

of identity, abundance, structure and activity of proteins expressed in a cell or organism, partly due to the lack of completed genomes or representative cDNA libraries from snakes (Serrano et al 2005).

Even though the number of proteins expressed in the snake venom glands is big, not all of the aforementioned hemostatically active components are present in the venoms of every snake species (Markland 1998c) and venom contents of venom batches from individual snakes within the same snake species are quite different (Daltry et al 1996).

Some snake venom metalloproteinases and serine proteinases affecting hemostasis and thrombosis are discussed in detail in the current work.

1.2.1. Snake venom metalloproteinases

More than hundred snake venom metalloproteinases, including isozymes from the same species, have been reported (Lou et al 2005). They belong to the reprolysin subfamily of zinc metalloproteinases that also includes ADAMs (A Disintegrin And Metalloproteinase) (Wolfsberg et al 1995a, Black and White 1998). The term reprolysin was proposed by Bjarnason and Fox in 1994 to refer to the fact that some members of the subfamily of metalloproteinases belong to reptiles (*rep*) whereas the others belong to the class of proteinases described in mammalian reproductive tissues (*repro lysin*). By now, more than 30 ADAM family members have been discovered in a variety of species and their different tissues (White 1995, Wolfsberg et al 1995a, 1995b; Wolfsberg and White 1996, Stone et al 1999, Primakoff and Myles 2000, Bridges and Bowditch 2005). ADAMs differ from the snake venom metalloproteinases in two important features: not all ADAMs are likely to have proteolytic activity and ADAMs have additional domains (an epidermal growth factor-like domain, a transmembrane domain and a cytoplasmic domain) not encountered in the structure of snake venom metalloproteinases (Jia et al 1996). ADAMs are key components in EGFR signalling and development, processing and shedding the ectodomains of membrane-anchored growth factors, cytokines and receptors. They have essential roles in fertilization, angiogenesis, neurogenesis, heart development and cancer (Blobel 2005).

1.2.1.1. Activity based classification of snake venom metalloproteinases

Based on the enzymatic activity, snake venom metalloproteinases can be divided into different groups.

1.2.1.1.1. Hemorrhagic metalloproteinases

Hemorrhagic activity is defined as the leakage of blood components through the basement membrane. It is caused by proteolytic degradation of the basement membrane proteins by the hemorrhagic proteinases (Bjarnason and Fox 1994, 1995, Bjarnason et al 1988, Baramova et al 1989). Hemorrhagic metalloproteinases are the most thoroughly studied proteins among snake venom metalloproteinases (reviews Bjarnason and Fox 1994, 1995, Matsui et al 2000, Gutierrez et al 2005 and many others). They are highly toxic, causing severe bleeding by acting on blood coagulation and hemostatic plug formation or by degrading the basement membrane or extracellular matrix proteins. They can act locally and/or systemically.

1.2.1.1.2. Nonhemorrhagic (weakly hemorrhagic) fibrin(ogen)olytic metalloproteinases

Fibrin(ogen)olytic enzymes break down fibrin-rich clots and help prevent further clot formation by their action on fibrinogen. Most of the metalloproteinases act fibrinogenolytically and fibrinolytically (Swenson and Markland 2005). Peptide bond cleavage by these enzymes is preferentially directed towards the Aα-chain and with

somewhat lower activity towards the Bβ-chain of fibrinogen (Markland 1998c). Examples of these enzymes are fibrolase from *Agkistrodon contortrix contortrix* (Ahmed et al 1990), lebetase from *Vipera lebetina* (Siigur and Siigur 1991), atroxase from *Crotalus atrox* (Tu et al 1996), neuwiedase from *Bothrops neuwiedi* (Rodrigues et al 2000, 2001, Izidoro et al 2003), LSF from *Lachesis stenophrys* (Leonardi et al 1999), B20 from *Bitis arietans* (Nok 2001) and leucurolysin-a (leuc-a) from *Bothrops leucurus* (Bello et al 2006).

However, there are some representatives among metalloproteinases, whose targets are preferentially Bβ-chains of fibrinogen. Examples are a proteolytic enzyme from the Taiwan Habu (*Trimeresurus mucrosquamatus*) (Sugihara et al 1985) and a proteolytic enzyme from *Vipera lebetina* venom (Gasmi et al 1991). The γ-chain does not appear to be a specific target as there are virtually no reports of fibrin(ogen)olytic snake venom metalloproteinases whose specificity is uniquely directed to this chain (Swenson and Markland 2005).

Fibrinolytic metalloproteinases (like fibrolase) without hemorrhagic activity or with weak hemorrhagic activity are good candidates for the development of drugs for clinical treatment of occlusive thrombi (Toombs 2001a, b).

1.2.1.1.2.1. Fibrin(ogeno)lytic metalloproteinase – lebetase – from *Vipera lebetina* venom

Lebetase is a metalloproteinase with fibrinolytic activity, which was isolated from the venom of *Vipera lebetina* (Levantine viper). Lebetase is a direct-acting fibrinolytic zinc metalloendopeptidase cleaving the Aα-chain of fibrinogen and more slowly the Bβ-chain. Lebetase has no effect on the γ-chain. Hydrolysis of fibrin by lebetase results in the degradation of the α- and β-chains, whereas the γ-γ-chains appears unaffected. The sites of cleavage of the oxidized B-chain of insulin by lebetase are Ala14-Leu15 and Tyr16-Leu17 (Siigur and Siigur 1991). Glucagon was a relatively poor substrate for lebetase; it was only slightly hydrolyzed at the position Tyr10-Ser11. Trummal et al. (2000) concluded on the basis of cleavage of 7-10 amino acid residues containing peptide fragments synthesized according to cleavage sites of the Aα-chain of fibrinogen for fibrolase (Retzios and Markland 1994) and for *Crotalus basiliscus basiliscus* enzymes (Retzios and Markland 1992) that lebetase hydrolyses the Aα-chain of fibrinogen at the positions Lys413-Leu414 and Pro516-Met517. The best peptide substrates for lebetase are substance P and peptide fragment of pregnancy zone protein (PZP), both are cleaved at the position Pro-Gln. Bradykinin and kallidin (Lys-bradykinin) are hydrolyzed exclusively at the Pro7-Phe8 bond. Lebetase has been found to remove a C-terminal dipeptide from bradykinin and kallidin. It is noteworthy that lebetase hydrolyzes bradykinin. This cleavage of bradykinin would destroy its vasodilatory action (Trummal et al 2000).

Lebetase is inhibited in normal human plasma. Saidi et al (1999) demonstrated that lebetase interacts with plasma proteinase inhibitors α2-macroglobulin and the α2M-related pregnancy zone protein. The proteolytic activity of lebetase against fibrinogen and azocasein could be inhibited by α2M. Lebetase activity is completely inhibited by EDTA, EGTA, DTT and 1,10–phenantroline. The metal ions Cd^{2+}, Cu^{2+}, Co^{2+} inhibit fibrinolytic and caseinolytic activity of lebetase (Siigur et al 1998b).

Polyclonal antibodies against lebetase cross-react with immunologically related proteinases from snake venoms (eg fibrolase from *Agkistrodon contortrix contortrix* venom) (Siigur et al 1996).

Judged from the degradation products, the cleavage specificity of fibrinogen for lebetase is broader than that for fibrolase.

1.2.1.1.3. Factor X activators

Factor X is a vitamin K-dependent blood coagulation factor. It is a glycoprotein of molecular mass ~59 kDa that is synthesized in the liver and secreted into blood as zymogen. Under physiological conditions, the factor X is activated by factor IXa, requiring Ca^{2+}, phospholipids, and factor VIIIa (a complex known as intrinsic Xase) or by factor VIIa

requiring Ca^{2+} and tissue factor (a complex known as extrinsic Xase). The activation results from the cleavage of the 52-residue activation peptide from the heavy chain of human factor X (Leytus et al 1984). The light chain of human factor X contains a number of γ-carboxyglutamic acid (Gla) residues forming the calcium-binding domain (Gla domain) of the molecule. Activators of blood coagulation factor X have been described in the venoms of many snakes (Yamada et al 1997, Morita 1998, Tans and Rosing 2001). The well-known factor X activator is the one isolated from the venom of Russell's viper (*Vipera russelli*) – RVV-X (Kisiel et al 1976, Takeya et al 1992). Structurally and functionally similar activator proteins have been found from *Bothrops atrox* (Hofmann and Bon 1987) and *Cerastes cerastes* venoms (Franssen et al 1983).

1.2.1.1.3.1. Factor X activator (VLFXA) from *Vipera lebetina* venom

The factor X activator (VLFXA) from *Vipera lebetina* venom has a heavy chain of 57.5 kDa and two light chains, 17.4 kDa and 14.5 kDa (reduced SDS-PAGE). Molecular masses of different VLFXA samples detected by MALDI-TOF analyses are at the interval of 85-91 kDa. The sugar content of VLFXA is about 15.5%. VLFXA exists in multiple isoforms (pIs between 5.3-6.6); the differences in isoelectric points are due, at least in part, to dissimilarities of the sialic acid content of respective isoforms. No functional differences have been discovered between any of the isoforms purified from different batches of the venom (Siigur et al 2001).

Based on results of hydrolysis of 6-9 amino acid residues containing peptide fragments synthesized according to the physiological cleavage region of human factor X and human factor IX, it has been found that VLFXA cleaves Arg-Ile bond in the nonapeptide NNLTR↓IVGG (I) and Arg-Val bond in the peptide NDFTR↓VVGG (II). VLFXA cleaves factor X fragment (I) more effectively than factor IX fragment (II). The activator converts the inactive human factor X to the active form of Xa in the presence of Ca^{2+} ions. VLFXA has no amidolytic activity against factor Xa substrates S-2337 [Benzoyl-Ile-Glu(Pip)-Gly-Arg-*p*NA-HCl] or S-2222 (Benzoyl-Ile-Glu-Gly-Arg-*p*NA-HCl), while *Vipera lebetina* venom itself hydrolyzes these substrates (Siigur et al 2001).

Hydrolysis of peptide substrates by VLFXA is inhibited with EDTA and *o*-phenanthroline. PMSF has no effect on the activity. The purified factor X activator has no effect on fibrinogen, prothrombin, plasminogen, indicating that it activates factor X specifically (Siigur et al 2001).

In conclusion, VLFXA hydrolyses human factor X at the position Arg52-Ile53. Factor X can be completely converted to factor Xa by VLFXA and the specific activity of factor Xa is the same as that of factor Xa obtained after activation by RVV-X. It seems that the catalytic mechanism of VLFXA should be similar to that of RVV-X (Morita 1998).

1.2.1.1.4. Prothrombin activators

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 venoms are rich sources of proteinases converting prothrombin to thrombin (Rosing and Tans 1991, Hutton and Warrell 1993, Kini 2005). Based on functional characteristics, structural properties and cofactor requirements, prothrombin activators have been categorized into four groups (groups A, B, C and D) (Rosing and Tans 1992, Kini et al 2001). The group A and B prothrombin activators are metalloproteinases and they are structurally unrelated to coagulation factors. The best-known activator belonging to the group A is ecarin from *Echis carinatus*, which 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).

1.2.1.1.5. Apoptosis inducing metalloproteinases

Endothelial cell apoptosis inducing metalloproteinases have also been found in various *Viperidae* and *Crotalidae* snake venoms (Fox and Serrano 2005). Examples are halysase from *Gloydius halys* venom (You et al 2003), agkistin from *Agkistrodon halys* (Wang et al 2003a), HV1 from *Trimeresurus flavoviridis* venom (Masuda et al 1998, 2001), VAP1 from *Crotalus atrox* venom (Masuda et al 2000), and graminelysin I from *Trimeresurus gramineus* venom (Wu et al 2001).

The apoptosis of endothelial cells induced by halysase is closely associated with activation of caspase-3 and decreased level of Bcl-X(L)/Bax. It was suggested that the proteinase domain and the disintegrin-like domain of halysase cooperatively contribute to the induction of endothelial cell apoptosis. Nevertheless, the apohalysase, which lacks metalloproteinase activity, is also able to induce the apoptosis (You et al 2003).

The caspase-3 activation and the Bcl-X(L)/Bax ratio alterations mediated apoptosis is induced by jararhagin from *Bothrops jararaca*, the protein with metalloproteinase, disintegrin-like and cycteine-rich domains. The apoptosis induction is associated with the enzymatic activity of the protein. Interestingly, the jararhagin is selective to endothelial cells (Tanjoni et al 2005).

In contrast, the apoptosis induced by BaP1 from *Bothrops asper*, the protein with metalloproteinase domain only, is independent of Bcl-X(L)/Bax, but is associated with caspase-8 activation and cellular FLICE-inhibitory protein up-regulation. Apoptosis is completely dependent on BaP1 enzymatic activity (Diaz et al 2003).

VAP1, the vascular apoptosis-inducing protein 1, has been proposed to provoke some caspase-independent apoptotic cell death pathway for human umbilical vein endothelial cells (HUVECs) (Maruyama et al 2005). Authors suggest that VAP1 induces apoptosis by activating some special signals, like “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. The targets of proteinase or disintegrin domain of the VAP1 are not clear (Maruyama et al 2005).

Graminelysin-1 from *Trimeresurus gramineus*, the protein with metalloproteinase domain only, induces apoptosis of HUVECs in a dose-dependent manner. It was suggested that the catalytic activity of graminelysin I on matrix proteins contributes to its apoptosis-inducing activity (Wu et al 2001).

1.2.1.1.5.1. Endothelial cell apoptosis inducing metalloproteinase (VLAIP) from *Vipera lebetina* venom

Endothelial cell apoptosis inducing metalloproteinase (VLAIP) from *Vipera lebetina* venom is a heterodimeric glycoprotein with molecular mass of 106 kDa (Siigur et al 2002). The proteinase hydrolyzes azocasein, fibrinogen and oxidized insulin B-chain. VLAIP readily cleaves the Aα-chain of fibrinogen and more slowly the Bβ-chain of fibrinogen, the γ-chain does not appear to be a target for hydrolysis. VLAIP does not cleave fibrin. The oxidized insulin B-chain cleavage by VLAIP differs from cleavages with lebetase. VLAIP hydrolyses only Ala14-Leu15 bond in the oxidized insulin B-chain. Bradykinin is cleaved at the position Pro4-Met5; the Aα-chain of fibrinogen at positions Lys413-Leu414 and Pro516-Met517 (Siigur et al 2002).

The enzyme is inhibited by EDTA or 1,10-phenanthroline. No inhibition has been detected with PMSF.

The effects of VLAIP on HUVECs are 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 which occurs due to apoptosis. VLAIP inhibits endothelial cell adhesion to

extracellular matrix proteins: fibrinogen, fibronectin, vitronectin, collagen I and collagen IV (Trummal et al 2005, paper VII).

1.2.1.2. Domain structure based classification of snake venom metalloproteinases

Based on the protein domain structure snake venom metalloproteinases are classified as follows: P-I (low molecular mass, 20-30 kDa) snake venom metalloproteinases having only a metalloproteinase domain; P-II (medium size, 30-60 kDa) snake venom metalloproteinases composed of a metalloproteinase domain and a disintegrin domain; P-III (high molecular mass, 60-100 kDa) snake venom metalloproteinases containing a metalloproteinase domain, a disintegrin-like domain and a cysteine-rich domain and P-IV snake venom metalloproteinases having the P-III structure and an additional C-type lectin-like domain connected by disulfide bonds (Bjarnason and Fox 1995). These snake venom metalloproteinases are synthesized in zymogen form from corresponding mRNAs (Fig. 3). The P-I class metalloproteinases are synthesized from N-I class polynucleotides encoding a signal peptide, a propeptide and metalloproteinase domains or from N-II class polynucleotides encoding after the metalloproteinase domain a disintegrin-like domain which will be posttranslationally processed to the metalloproteinase and disintegrin. In some cases, N-II class gives P-II proteins as well. P-III proteinases are synthesized from N-III polynucleotide class encoding a signal peptide, a propeptide, a metalloproteinase, a disintegrin-like and cysteine-rich domains (Bjarnason and Fox 1998). The synthesis scheme of P-IV class proteinases has been controversial. There is no evidence for the N-IV polynucleotide class. P-IV class proteinases are synthesized as P-III ones (heavy chain) and the light chain(s) are synthesized from an individual gene and connected with P-III protein posttranslationally as shown in the current work (Siigur et al 2004, paper VI).

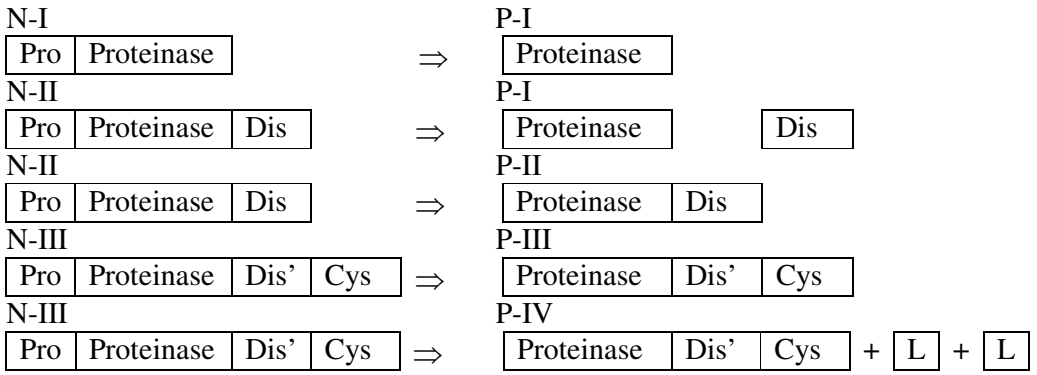

Figure 3. Schematic representation of metalloproteinase classes P-I – P-IV and their synthesis from corresponding polynucleotides. Pro – signal and propeptide region, Dis – disintegrin region, Dis' – disintegrin-like region, Cys – cysteine-rich region, L – lectin domain.

The biological functions of distinct domains of multidomain metalloproteinases are not fully clear. The proteolytic activity is exhibited by the metalloproteinase domain. The disintegrin-like domain could target the proteins to the specific cell surface protein integrin, and block the binding of normal ligands to the integrins. The cysteine-rich domain probably influences the composite activity of the protein and its role in a particular protein could depend on other domains (Fox and Serrano 2005); however, the toxic effects of P-III proteins are most likely dependent on the cysteine-rich domain of the protein (Ramos and Selistre-de-Araujo 2006). C-type lectins bind selectively to platelet membrane proteins or blood coagulation factors (Kini and Evans 1990) and are therefore involved in the targeting of the protein.

Snake venom metalloproteinases are zinc metalloproteinases, which contain Zn^{2+}-binding motif of HEXXHXXGXXH, followed by conserved aspartic acid (typical of

reprolysins). They belong to the metzincin superfamily, as they all possess a methionine containing turn of similar conformation (the Met-turn, CI/VM; Fig. 4) (Bode et al 1993, Hooper 1994). The reprolysin family lacks the fifth zinc ligand (tyrosine in astacin and serralysin subfamilies), leaving the zinc atom tetrahedrally co-ordinated (three histidines and water anchored to glutamic acid residue) (Bode et al 1993, Gomis-Rüth et al 1993). The molecule globular packing stability and holding of N- and C-terminus in close proximity is achieved by a calcium ion liganded at the opposite side of the active site (Bode et al 1994). The proteolytic as well as the hemorrhagic activity of these enzymes can be inhibited through Zn^{2+} ion chelation with chelating agents like EDTA.

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FIBROLASE       HELGHNLGMNHDGNQCHCGANSCVMAAML
LEBETASE        HELGHNLGMNHDGDQCHCGANGCVMSVVL
ATROXASE        HELGHNLGMNHDGEKCLRGASLCIMRPGL
ATROLYSIN       HELGHNLGMEHDGKDCLRGASLCIMRPGL
ADAMALYSIN      HELGHNLGMEHDGKDCLRGASLCIMRPGL
NEUWIEDASE      HELGHNLGMEHDGKDCLCGASLCIMSPGL
                          :    :: ::   :: : :::
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Figure 4. Sequence homology in the active site of fibrinolytic and hemorrhagic metalloproteinases by Swenson and Markland (2005). Differences are marked by dots.

Snake venom metalloproteinases are synthesized and stored in the venom gland as inactive precursors (Hite et al 1992). The cysteine switch-like motif PKMCGV in the prosequence of the zymogen is responsible for blocking the active site by binding via the cysteinyl sulfur to the active site Zn^{2+} ion and preventing proteolytic activity of the zymogen. After secretion from the gland, the proteolytic processing converts the zymogen to the active enzyme by removing this thiol group (Grams et al 1993).

The inhibition of snake venom metalloproteinases (and the protection against their own toxicity) can be achieved, in addition to inactive zymogen synthesis, through high concentrations of citrate in venoms that inhibit enzymes by metal-ion chelation (Francis et al 1992) or through inhibitory peptides present in venoms (Francis and Kaiser 1993). Upon dilution, for example, when venom is injected into prey, citrate and peptide inhibitors dissociate from the proteinase and allow activation (Francis and Kaiser 1993). Another way for natural inhibition of snake venom metalloproteinases (especially in the case of snake venom hemorrhagic metalloproteinases) is their activity neutralization by snake serum glycoproteins that form a complex with the enzyme (Weissenberg et al 1991).

1.2.1.3. Structure-function relationship of snake venom metalloproteinases

Due to a wide variety of different activities of snake venom metalloproteinases within a group of similar enzymes, the question of the relationship between their structure and their function has arisen. The elucidation of structures of these proteolytic enzymes would help us determine their structure-function relationships and expand their potential pharmacological application.

A majority of snake venom metalloproteinases are hemorrhagic, but some of them, particularly those belonging to the P-I class (fibrolase, lebetase, atroxase, neuwiedase, leuc-a), are devoid of hemorrhagic activity (Randolph et al 1992, Markland 1996, Siigur and Siigur 1991, Willis et al 1988, Rodrigues et al 2000, Bello et al 2006). Many attempts have been made to estimate the hemorrhagic potential of snake venom metalloproteinases and to define molecular characteristics related to this biological activity (Paine et al 1992, Manning 1995, Takeya et al 1989, 1990a, 1990b, Gasmi et al 2000, Tsai et al 2000, Bolger et al 2001). An increasing number of sequences and structures of snake venom metalloproteinases has proven some earlier hypothesis to be wrong (Ramos and Selistre-de-Araujo 2004).

Gasmi et al (2000) identified residues, which he proposed to be characteristic of hemorrhagic metalloproteinases only, and other residues shared by non-hemorrhagic ones. Other authors (Ramos and Selistre-de-Araujo 2004, Lou et al 2005) did not find any specific residues when comparing the amino acid sequences of non-hemorrhagic and hemorrhagic proteinases. Garcia et al (2004) proposed that hemorrhagic activity of snake venom metalloproteinases depends on post-translational modifications, such as glycosylation, whereas their proteolytic activity is not dependent on such modifications. Another explanation for the hemorrhagic activity of snake venom metalloproteinases could be associated with the molecular surface properties (Ramos and Selistre-de-Araujo 2004). You et al (2006) hypothesized that two distinct domains, the metalloproteinase and the disintegrin-like domains, of the high molecular weight metalloproteinase could cooperatively contribute to the hemorrhagic function of the enzyme. The support for the hypothesis can be driven from the reports of higher hemorrhagic activity of P-III class metalloproteinases compared to P-I class (Matsui et al 2000).

Today, several crystal structures of P-I class snake venom metalloproteinases are available (Gomis-Rüth et al 1993, Kumasaka et al 1996, Gong et al 1998, Zhu et al 1999, Huang et al 2002, Watanabe et al 2003, Lou et al 2005). All these crystal structures are of the shape of an oblate ellipsoid with a central five-stranded β-sheet mixed with α-helices along with the conserved Met-turn structure (Fox and Serrano 2005, Lou et al 2005). The different number of cysteinyl residues present in the sequences of P-I class metalloproteinases along with alternative disulfide bond patterns would lead to a possibility for different tertiary structures, which is perhaps related to structural stability and/or post-translational processing (Fox and Serrano 2005). Attempts to correlate biological activities with tertiary structures have not been resultful yet, but it is likely that the additional crystal structures, especially those of members from other classes, could expand our understanding of the biological functions of snake venom metalloproteinases.

1.2.2. Snake venom serine proteinases

Snake venom serine proteinases are among the best-characterized venom enzymes affecting the hemostatic system. They act on macromolecular substrates of the coagulation, fibrinolytic, and kallikrein-kinin systems, and on platelets, causing an imbalance of the hemostatic system of the prey. Currently, more than fifty amino acid sequences of serine proteinases are available in the databanks (Serrano and Maroun 2005). They are found in venoms of the snake families *Viperidae*, *Crotalidae*, *Elapidae* and *Colubridae*. Both acidic and basic serine proteinases have been isolated from snake venoms. Basic serine proteinases exhibit direct platelet-aggregating activity, while acidic proteinases have been described to act proteolytically on a variety of substrates related to hemostasis (Serrano and Maroun 2005).

1.2.2.1. Activity based classification of snake venom serine proteinases

According to the action spectrum, snake venom serine proteinases can be divided into different groups.

1.2.2.1.1. Fibrinogenolytic serine proteinases

The group consists of enzymes with fibrinogenolytic activity. They specifically cleave preferentially either fibrinopeptide A (venombins A) or fibrinopeptide B (venombins B), or both A and B (venombins AB) from the N-terminal part of fibrinogen, promoting coagulation (review Serrano and Maroun 2005). These enzymes have been called thrombin-like, because of their ability to clot fibrinogen similarly to α-thrombin (mimetic of one activity among several thrombin's activities) (reviews Markland 1998c, Pirkle 1998). Unlike thrombin, snake venom serine proteinases do not activate coagulation factor XIII (example of the exception is contortrixobin from the venom of *Agkistrodon contortrix contortrix* (Amiconi et al 2000), but the activation rate is 250-500-fold lower than that of thrombin) and fibrin

monomers produced are not cross-linked, allowing rapid dissolution of clots formed (Stocker et al 1982).

A majority of these fibrinogenolytic enzymes cleave only fibrinopeptide A (FPA) from fibrinogen. Representatives of FPA releasing serine proteinases are acutobin from *Deinagkistrodon acutus* (Wang et al 2001), ancrod from *Calloselasma rhodostoma* (Nolan et al 1976, Bell 1997), batroxobin from *Bothrops atrox* (Stocker and Barlow 1976, Itoh et al 1987), calobin from *Agkistrodon caliginosus* (Hahn et al 1996) and crotalase from *Crotalus adamanteus* (Markland 1976), while an example of a preferentially fibrinopeptide (FPB) releasing enzyme is contortrixobin from *Agkistrodon contortrix contortrix* (Amiconi et al 2000). Gabonase from *Bitis gabonica* (Pirkle et al 1986) and bilineobin from *Agkistrodon bilineatus* (Nikai et al 1995) release both fibrinopeptides A and B.

In addition to coagulant fibrinogenases, some venoms contain enzymes cleaving the C-terminal part of the Bβ-chain of fibrinogen converting it uncoagulable by thrombin. These so-called beta-fibrinogenases have been characterized in the venoms of *Trimeresurus mucrosquamatus* (Hung et al 1994) and *Trimeresurus stejnegeri* (Gao et al 1998).

1.2.2.1.1.1. Fibrinogenolytic serine proteinases from *Vipera lebetina* venom

Two glycosylated serine fibrinogenases have been isolated from *Vipera lebetina* venom. α-Fibrinogenase (VLAF) has a molecular mass of 31.1 kDa and an isoelectric point >10. VLAF cleaves casein, insulin and fibrinogen, but not low molecular weight esters of arginine, lysine or tyrosine (Mähar et al 1987, Samel et al 2002). β-Fibrinogenase (VLBF) with molecular mass of 42.2 kDa, is in contrast acidic in its native form (pI<3) (Siigur et al 1991, Samel et al 2002). β-Fibrinogenase is a typical arginine esterase, which hydrolyses esters and amides of arginine and cleaves the Bβ-chain of fibrinogen. VLBF is highly glycosylated (32.8%), which makes the enzyme extremely thermostable (Siigur et al 1991, Samel et al 2002). VLAF is less glycosylated (15.1%) and accordingly rather thermolabile (Mähar et al 1987, Samel et al 2002).

1.2.2.1.2. Factor V activating serine proteinases

Factor V is a protein cofactor of the coagulation system without enzymatic activity. It is a glycoprotein of molecular mass ~330 kDa. In normal physiological conditions the factor V is activated by thrombin to factor Va. Snake venoms contain enzymes acting similarly to thrombin in activation of factor V. Representatives of factor V activating snake venom serine proteinases are RVV-V from *Vipera russelli* (Tokunaga et al 1988), which activates factor V to the same extent as α-thrombin and contortrixobin, which was reported to activate factor V to a lower extent than thrombin (Amiconi et al 2000). Factor V activating proteins have also been detected in *Elapidae* venoms (Rosing et al 2001).

1.2.2.1.2.1. Factor V activator (VLFVA) from *Vipera lebetina* venom

A specific factor V activating serine proteinase (VLFVA) has been found in *Vipera lebetina* venom (Siigur et al 1998a). VLFVA with a molecular mass of 28.4 kDa, as determined by matrix assisted laser desorption ionization time-of-flight mass-spectrometry (MALDI-TOF MS), is a single-chain glycoprotein. The protein comprises 11.1% carbohydrates. Besides factor V, no other protein substrate for VLFVA has yet been identified. VLFVA hydrolyzes several synthetic arginine ester substrates, such as benzoylarginine ethyl ester (BAEE), tosylarginine methyl ester (TAME) and amide substrate such as Pro-Phe-Arg-MCA. The arginine ester hydrolase activity of the enzyme is remarkably lower than that of the crude venom. The ability of VLFVA to activate factor V and its activity to BAEE and TAME are inhibited by the serine proteinase inhibitor, diisopropylfluorophosphate (DFP) (Siigur et al 1998a).

It should be mentioned that thrombin mediated factor V activation occurs as a result of three cleavages in the factor V molecule, whereas the proteolytic activation of factor V by

RVV-V and VLFVA involves cleavage at only one site (Arg1545-Ser1546) (Siigure et al 2001, paper IV).

1.2.2.1.3. Protein C activating serine proteinases

Protein C is a vitamin K-dependent glycoprotein, which in its activated form degrades factor Va (the activated form of factor V) and has therefore anticoagulant activity (Stenflo 1976). An example of snake venom serine proteinase with the capability of activating protein C is ACC-C from *Agkistrodon contortrix contortrix* (Kisiel et al 1987, McMullen et al 1989).

1.2.2.1.4. Platelet aggregation inducing serine proteinases

This group includes snake venom serine proteinases directly activating platelet aggregation and thus having activity typical of α-thrombin. Among these proteins is thrombocytin from *Bothrops atrox* (Niewiarowski et al 1979, Kirby et al 1979), which causes platelet aggregation, platelet serotonin release and activation of coagulation factor XIII by limited proteolysis. Representatives of this group are also crotalocytin from *Crotalus horridus horridus*, which induces platelets aggregation and ATP secretion from platelets (Schmaier and Colman 1980), MSP 1 from *Bothrops moojeni*, which induces platelet aggregation and enhances ADP-induced aggregation (Serrano et al 1993) and cerastobin from *Cerastes vipera*, which exhibits a proteolytic activity towards protein constituents of the platelet cytoskeleton, and its activation of human platelets is not related to ADP release and/or prostaglandin synthesis (Farid et al 1990). Representatives are also cerastocytin from *Cerastes cerastes*, which is a potent platelet activator with the ability to activate factor XIII (Marrakchi et al 1995) and bothrombin from *Bothrops jararaca*, which is able to cause platelet aggregation in the presence of exogenous fibrinogen (Nishida et al 1994).

1.2.2.1.5. Kallikrein-like serine proteinases

The group of serine proteinases includes so-called “kallikrein-like” enzymes, which release hypotensive Lys-bradykinin or bradykinin from kininogen. Enzymes crotalase (Markland 1976), KN-BJ (Serrano et al 1998) and elegaxobin II (Oyama et al 2003) exhibit both kinin-releasing and coagulant activities. Halystase is a bradykinin-realeasing enzyme devoid of coagulant activity but cleaving fibrinogen chains (Matsui et al 1998). Bradykinin releasing enzyme has been isolated from the *Vipera lebetina* venom as well (Siigur et al 1982).

1.2.2.1.6. Plasminogen activating serine proteinases

Some snake venoms contain specific serine proteinases that convert plasminogen to plasmin. Liberated plasmin degrades fibrin. Plasminogen activator TSV-PA has been purified from *Trimeresurus stejnegeri* venom and the enzyme has been crystallised (Zhang et al 1995, 1997, Parry et al 1998). The activator haly-PA has been found in *Agkistrodon halys* venom (Park et al 1998).

1.2.2.1.7. Prothrombin activators homologous to blood coagulation Factor Xa

In addition to snake venom metalloproteinases activating prothrombin to thrombin and belonging to groups A and B of prothrombin activators as mentioned above, snake venom serine proteinases activating prothrombin to thrombin are classified into the groups C and D.

Group C and group D serine proteinases have been exclusively found in the venoms of the Australian elapids (Rosing and Tans 1991, Rosing and Tans 1992, Rao et al 2003, Kini 2005). Examples are the following: pseutarin C from *Pseudonaja textilis* – a representative of group C activator (Rao and Kini 2002) and trocarin D from *Tropidechis carinatus* – the best studied group D prothrombin activator (Joseph et al 1999, Joseph and Kini 2001).

In conclusion, it should be mentioned that the classification of snake venom serine proteinases into groups as described above is rather artificial, because some serine proteinases have more than one activity, thus belonging simultaneously to different groups, but nevertheless none of the enzymes referred to exhibits all the multiple functions of thrombin.

1.2.2.2. Structure of snake venom serine proteinases

Isolated snake venom serine proteinases are one-chain proteins with molecular masses between 26 and 67 kDa, depending on their carbohydrate content. Only one two-chain serine proteinase with fibrinolytic activity – brevinase from the venom of *Agkistrodon blomhoffii brevicaudus* – has been reported (Lee et al 1999). cDNA cloning results suggested that the two different chains of the brevinase might be translated from a single transcript and cleaved by posttranslational processing (Lee and Park 2000).

cDNA cloning and sequencing of snake venom serine proteinases has revealed that these proteins contain open reading frames (ORF) of ~800 bp, short 5'-untranslated regions (UTRs) and long 3'-UTRs of variable length (up to more than 1200 bp). Snake venom serine proteinases are synthesized as zymogens of ~256-257 amino acids with a putative signal peptide of 18 amino acid and proposed activation peptide of six amino acid residues (QKSSEL) (Serrano and Maroun 2005). Most of the snake venom serine proteinases are likely to be glycosylated via a variable number of *N*- or *O*-glycosylation sites encountered in the sequences of proteinases. *O*-glycosylation sites are rather rare in snake venom serine proteinase sequences. Examples of proteins with *O*-glycosylation sites are PA-BJ (with platelet aggregating activity) from *Bothrops jararaca* (Serrano et al 1995) and *Bothrops* protease A (BPA) from the same venom (Murayama et al 2003).

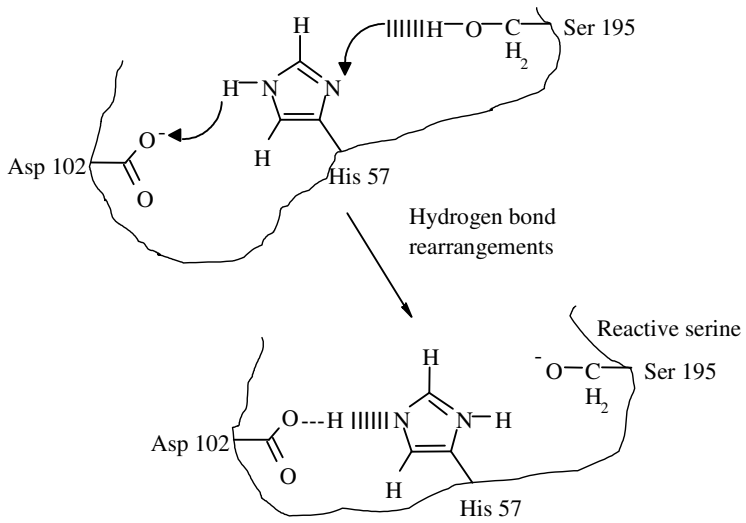

Figure 5. The active site of serine proteinases. The aspartic acid side chain (Asp 102) induces the histidine (His 57) to remove the proton from the serine 195. This activates the serine proteinase to form a covalent bond with the enzyme substrate, hydrolyzing a peptide bond (Alberts et al 2002).

The conserved active site of snake venom serine proteinases includes a highly reactive serine residue (Ser195), histidine (His57) and aspartic acid (Asp102) (catalytic triad) (Fig. 5). Serine proteinases have been classified into evolutionarily and structurally unrelated clans. "Clan" is the term used to describe a group of families, the members of which have evolved from a single ancestral protein but have diverged so far that we can no longer prove their relationship by comparison of the primary structures (Barrett and Rawlings 1995). Enzymes with the order of catalytic triad His, Asp, Ser belong to the clan SA of serine peptidases; snake venom serine proteinases are members of the trypsin family S1 of the clan

SA (Halfon and Craik 1998). The fourth residue with side chains in the active site – Ser214 – appears to have been conserved in chymotrypsin-like proteinases. This residue is required for substrate recognition, ensuring optimal diffusion into the active site (Krem et al 2002).

Besides phenylmethylsulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP), which modify the active serine residue, snake venom serine proteinases are competitively inhibited by benzamidine and p-aminobenzamidine. In general, snake venom serine proteinases are insensitive to endogenous serine proteinase inhibitors (serpins) (Serrano and Maroun 2005).

Snake venom serine proteinases contain twelve cysteine residues, ten of which form five disulfide bonds, based on homology with trypsin (Itoh et al 1987); the remaining two cysteines (Cys91-Cys245e) form a unique and conserved bond among serine proteinases, connecting the highly conserved seven residue C-terminal extension with the main body of these enzymes (Parry et al 1998). Another characteristic feature of the snake venom serine proteinases is a salt bridge between the N-terminal amino group of the first valine and the side chain of Asp194, which precedes the catalytic Ser195. This salt bridge is believed to stabilize the structure of the active site (Zhang et al 1997, Henschen-Edman et al 1999). Asp189 is conserved in practically all trypsin-like serine proteinases; it is located at the bottom of a "specificity pocket" near the active site (Henschen-Edman et al 1999). This residue forms a salt bridge with the basic residue of the scissile bond of the substrate. All trypsin-like enzymes (except plasminogen activator TSV-PA) possess a Gly193 at the S1 subsite (Zhang et al 1997, Parry et al 1998). The important role of residue 193 of snake venom serine proteinases for substrate and inhibitor specificity has been demonstrated by several authors (reviewed by Serrano and Maroun 2005).

1.2.2.3. Putative origin of snake venom serine proteinases

Snake venom serine proteinases display high levels of sequence identity, despite having different but specific physiological properties, suggesting that they diverged from a common ancestral molecule, leading to the establishment of a gene family (Braud et al 2000). Analysis of the batroxobin gene organization (Itoh et al 1988) has revealed that exon/intron structure of batroxobin is similar to the kallikrein gene, but not to the prothrombin gene. Based on these results, it was suggested that the snake venom serine proteinases have evolved from glandular kallikrein and their functions have changed during evolution. Observation of venom gland serine proteinases has shown that nucleotide substitutions in the protein-coding region have occurred more frequently compared to UTRs, that non-synonymous nucleotide substitutions causing amino acid changes occur much more frequently in venom gland serine proteinase genes than in general isozyme genes, and that introns are highly conserved, like their UTRs, suggesting that only the mature protein-coding regions have evolved at an unusually higher rate accompanied by diversification of their products leading to different physiological functions (Deshimaru et al 1996).

Furthermore, snake venom serine proteinases and trypsin have a common three-dimensional structure which refers to the trypsin/kallikrein gene family and not to the thrombin gene family (Zhang et al 1997). One common feature suggesting the origin of snake venom serine proteinases from the glandular kallikrein is a conserved Pro219 among all snake venom serine proteinases just like in the molecule of glandular kallikrein (Serrano and Maroun 2005).

1.2.2.4. Structure-function relationship of serine proteinases

As already mentioned in the case of metalloproteinases, the question of the relationship between the structure and the function of snake venom serine proteinases, similar enzymes with a wide variety of different activities, has arisen. Based on experimental data gathered so far, the primary sequence similarity or dissimilarity of serine proteinases is not sufficient to explain the specificities of certain enzymes. The determinants of biorecognition may involve the contribution of many residues/factors (Amiconi et al 2000, Jiao et al 2005).

For example, the loop 95-98 (Chymotrypsinogen numeration) in the spatial proximity of the active site may be essential for the substrate specificity (Zhang et al 1997). Remarkable differences in substrate specificities of considerably homologous snake venom serine proteinases (homology 60-70%) should depend on the surrounding and the arrangement of the active site cleft. Different specificities occur not only in the case of protein substrates but also with low-molecular peptide substrates (Meier et al 1985). On the other hand, the specificity and activity of serine proteinases can be affected by carbohydrate moieties of the enzyme (Serrano and Maroun 2005, Zhu et al 2005). Additional studies have to be carried out to gain more information about crystal structure of these enzymes, which all together would lead to solving the paradox how the enzymes with such a high similarity show such an exquisite specificity toward different substrates (Serrano and Maroun 2005).

1.3. ORIGIN AND EVOLUTION OF SNAKE VENOMS

Despite lots of information based on the classical morphological analysis and analysis of snake phylogeny including the data concerning homology of the venom-secreting glands, still much less is known about how the biochemical arsenal of snakes was assembled in the course of evolution (Fry and Wüster 2004). The venom content analysis including amino acid/nucleotide sequence analysis could be a valuable tool to overcome speculations related to the origin and evolution of venom-secreting glands. The first attempts (Fry and Wüster 2004) to correlate snake venom protein sequences with the evolution of venom supported the idea that the venom evolved once, at the base of the advanced snake radiation, rather than multiple times in different lineages. According to analysis of toxin families, authors proposed that some of them (Kunitz-type protease inhibitors, CRISPs (cysteine-rich secretory proteins), galactose-binding lectins, M12B peptidases, nerve growth factors) were present in the venom before the diversification of advanced snakes. The phospholipase A_2 and natriuretic toxin families seemed to be recruited independently in elapids and viperids: for instance C-type lectins were shown to be in *Viperidae* only, with the α-chains and β-chains resulting from an early duplication event. Additional analysis of snake venom toxins and related nonvenom proteins revealed that toxin recruitment events have occurred at least 24 times in the evolution of snake venoms. Besides the gene recruitment events, the modifications of existing salivary proteins have given raise to some toxins (CRISP and kallikrein toxins; the evolution of the latter ones has been discussed in connection with serine proteinases above). Furthermore, the toxin types, where the ancestral protein was strongly cysteine cross-linked, flourished into functionally diverse, novel toxin multigene families (Fry 2005).

2. MATERIALS AND METHODS

The cDNA library was constructed from the mRNA isolated from the venom gland of one adult Central Asian *Vipera lebetina* snake three days after milking. The library was found to contain $5.5x10^6$ recombinants.

The methods used are described in detail in the publications of this thesis.

The methods are the following:

cDNA library construction (I)
cDNA library screening with a labelled probe (I, VI)
Design of primers for PCR amplification (I, II, III, V, VI, VII)
DNA cloning (I, II, III, V, VI, VII)
DNA sequencing (I, II, III, V, VI, VII)
mRNA isolation (I)
PCR amplification (I, II, III, V, VI, VII)
PCR product purification (I, II, III, V, VI, VII)
Sequence analysis (I, II, III, V, VI, VII)
Synthesis of primers for PCR amplification (I).

All nucleic acid sequences determined by us and reported in this thesis are available from GenBank under the following accession numbers:
AY987816 – lebetase isoform Le-4
X97894 – lebetase isoform Le-3
AY339162 – factor X activator (VLFXA), heavy chain (HC)
AY339163 – factor X activator (VLFXA), light chain 1 (LC1)
AY578116 – factor X activator (VLFXA), light chain 2 (LC2)
AY835997 – endothelial cell apoptosis inducing metalloproteinase monomer B (VLAIP-B)
AY835996 – endothelial cell apoptosis inducing metalloproteinase monomer A (VLAIP-A)
AF163973 – factor V activator (VLFVA - VLP1)
AJ251153 – homolog of factor V activating enzyme (VLP2)
AF536235 – β-fibrinogenase (VLBF)
AF528193 – α-fibrinogenase (VLAF).

3. RESULTS AND DISCUSSION

To avoid any uncertainties in the classification of venomous snakes, the term "*Viperidae* snake" is hereafter used for the following genera: *Bitis, Cerastes, Echis, Eristicophis* and *Vipera.* Accordingly the Latin name used for Levantine viper is *Vipera lebetina* (not *Macrovipera lebetina*) and for Russell's viper *Vipera russellii* (not *Daboia russelii*).

Vipera lebetina (Levantine viper) is a snake found in the South-Eastern Europe, in the South-Western Asia and in the North-Western Africa. Its venom comprises serine proteinases (α-fibrinogenase, β-fibrinogenase, factor V activator, kininogenases) and metalloproteinases (fibrinolytic enzyme lebetase, factor X activator), which affect coagulation and fibrinolysis. Most of the proteinases found in the venom of *Vipera lebetina* (except lebetase) are known to be glycosylated.

3.1. METALLOPROTEINASES FROM THE *VIPERA LEBETINA* VENOM (IV, VII)

Metalloproteinases found in *Vipera lebetina* venom comprise hemorrhagic and nonhemorrhagic enzymes (including fibrinolytic enzyme – lebetase) (Siigur and Siigur 1991, 1992, 2004), factor X activator (VLFXA) (Siigur et al 1995, 2001) and endothelial cell apoptosis inducing metalloproteinase.

Lebetase, a metalloproteinase with thrombolytic activity, is a direct-acting fibrinolytic enzyme which cleaves directly fibrin, but has no effect on plasminogen activation. The enzyme readily hydrolyzes the α-chain and more slowly the β-chain of fibrinogen. Lebetase has very low hemorrhagic activity. Besides, lebetase is an anticoagulant enzyme inhibiting platelet aggregation (Siigur et al 1998b).

Vipera lebetina venom contains components, which are able to activate factor X (Siigur et al 1995). Factor X, a vitamin K-dependent protein, converges extrinsic and intrinsic pathways of the coagulation cascade in normal conditions and therefore settles in a central position in the blood coagulation process. Activation of human factor X to factor Xa, a serine proteinase, results in cleavage of a 52-residue activation peptide from the heavy chain of factor X (Kisiel et al 1976).

An endothelial cell apoptosis inducing metalloproteinase was also isolated from the venom of *Vipera lebetina.* Endothelial cells play a critical role in vascular hemostasis, such as blood coagulation, vascular contraction and the control of vascular permeability. In addition to endothelial cells apoptosis induction, this metalloproteinase readily hydrolyzes the Aα-chain and more slowly the Bβ-chain of fibrinogen, but does not cleave fibrin.

3.1.1. Lebetase (I and VIII)

Lebetase, a non-glycosylated direct-acting fibrinolytic enzyme, has at least four isoforms with pIs in the interval of 4.6-5.6, whose cleavage products of fibrinogen are identical. Lebetase isoform with pI 5.3 and molecular mass of 22,890 Da (corrected value) (clone Le-3) is the first fibrinolytic enzyme whose nucleic acid sequence was determined from *Viperidae* venom. The knowledge of lebetase isoforms and the synthesis of disintegrins were expanded by cloning an additional clone – Le-4 – a novel lebetase isoform. The novel isoform has a calculated molecular mass of 22,930 Da and pI 5.6.

3.1.1.1. Description of sequences

The lebetase isoform clone Le-3 cDNA consists of 2011 base pairs, including a 5'-untranslated region, an open reading frame of 1434 bp and a 3'-untranslated region (Table 1). The deduced amino acid sequence from this open reading frame (478 amino acids) is much larger than would be necessary to encode a 22.9 kDa protein. The open reading frame consists of a signal peptide region (18 amino acids), a proregion (175 amino acids), a

metalloproteinase region (204 amino acids), and a carboxyterminal peptide [81 amino acids, including a spacer region (18 amino acids) and a disintegrin-like region (63 amino acids)], which must be posttranslationally removed (Fig. 7).

The nucleotide sequence of an another isoform of lebetase (1112 bp) – designated as a clone Le-4 – starts about 30 nucleotides upstream of the metalloproteinase coding region (verified by 4 independent clones) and comprises a metalloproteinase coding region (203 amino acids) and a 3'-untranslated region (Table 1). Differently from the lebetase clone Le-3, the clone Le-4 lacks the disintegrin-like domain coding region (Fig. 7). The translated protein sequence of Le-4 (amino acids 20-42) is in concordance with the amino acid sequence determined by direct sequencing of CNBr fragment, with one exception (N42-I42).

Comparison of the nucleotide sequence of the lebetase clone Le-3 (2011 bp) with 1794 bp and 1771 bp cDNA sequences of metalloproteinases ACLPREF and ACLPREH from *Agkistrodon contortrix laticinctus* (Selistre de Araujo and Ownby 1995) revealed 85.7% and 84.1% identities. Comparison of the deduced amino acid sequences of these clones showed 74.0% and 70.4% identity over 415 amino acid overlap. The deduced mature protein sequence revealed similarity with other snake venom metalloproteinases, particularly with fibrinolytic metalloproteinases [fibrolase 63% (Randolph et al 1992), atroxase 59.8% (Baker et al 1995)] but also with hemorrhagic and nonhemorrhagic proteinases (50-60%) [adamalysin (Gomis-Rüth et al 1994), HR1B (Takeya et al 1990a), HR2A (Miyata et al 1989), HR2 (Takeya et al 1989), HRTD (Hite et al 1994)].

In comparison with cDNA regions coding the mature protein, the 5'- and 3'-untranslated regions and the proenzyme regions of the cDNA sequence of lebetase clone Le-3 have a higher degree of similarity with corresponding regions of other snake venom metalloproteinases. Untranslated sequences of the lebetase clone Le-3 cDNA have 85-90% identity with untranslated cDNA regions of ACLPREF and ACLPREH from *Agkistrodon contortrix laticinctus* (Selistre de Araujo and Ownby 1995). The Le-3 contains a signal peptide (amino acids 1-18) similar to ACLPREF and ACLPREH (Selistre de Araujo and Ownby 1995). Extensive similarities are observed in the proprotein region. The variability is higher in the domains present on the mature protein.

The translated sequence of the clone Le-4 is 87% identical to Le-3 and 83% identical to a fibrinolytic enzyme from Tunesian *Vipera lebetina* venom (Gasmi et al 2000). The clone Le-4 shares high sequence identity with several other reprolysins [MP-4 (Francischetti et al 2004), MT-d (Jeon and Kim 1999), brevilysin L6 (Terada et al 1999), contortrostatin (Zhou et al 2000), fibrolase (Randolph et al 1992), BaP-1 (Watanabe et al 2003)].

3.1.1.2. Characterization of the enzymes

As mentioned above, the open reading frame of the lebetase clone Le-3 consists of a signal peptide region, a propeptide region, a metalloproteinase domain, and a disintegrin-like domain. The conserved PKMCGV sequence ("cysteine switch" motif) between the signal sequence and the mature protein was identified in the deduced amino acid sequence. As all Le-4 clones were truncated from the 5'-end and started only 30 nucleotides upstream the metalloproteinase coding region, both the conserved PKMCGV and the signal peptide region were missing (Fig. 7).

As typical representatives of the reprolysin family, the deduced amino acid sequences of both lebetase isoforms (the clone Le-3 and the clone Le-4) have the zinc-binding motif **H**ELG**H**NLGMN**H** or **H**EIG**H**NLGMD**H,** respectively, in the catalytic domain, followed by aspartic acid. In the disintegrin-like domain of the clone Le-3, the RGD sequence is replaced by VGD motif (Fig. 7).

Both isoforms contain six conserved cysteines that form three disulfide bonds, thus belonging to the three-disulfide bond proteinase class. The conserved pattern of disulfide bonds is the same as determined for fibrolase (Manning 1995).

The P-I class of metalloproteinases may have a variable number of cysteinyl residues in the protein sequence (Fox and Serrano 2005). The majority of the reprolysins are three-disulfide proteinases (Bjarnason and Fox 1995). Fibrinolytic enzymes atroxase (Baker et al

1995), nonhemorrhagic adamalysin II (Gomis-Rüth et al 1993) and hemorrhagic atrolysin C (Fox and Bjarnason 1995) contain two disulfide bonds.

Cysteinyl residues in the positions 233 and 235, as well as in the positions 222 and 241 (numbering by Fox and Serrano 2005) are critical for the maintenance of the protein structure (Fox and Serrano 2005). The lack of cysteinyl residues in the positions 233 and 235 leads to the proteolytic processing of the disintegrin-like domain from the metalloproteinase domain. The presence of cysteinyl residues in the positions 222 and 241 plays a role in arresting the proteolytic processing of the disintegrin-like domain from the metalloproteinase domain to maintain a two-domain structure comprised of the metalloproteinase and the disintegrin-like domain (Fox and Serrano 2005). The Le-3 clone of a lebetase, as well as contortrostatin (Zhou et al 2000) and acostatin-B (Okuda et al 2002) lack cysteines at these positions, and the spacer region and the disintegrin-like domain are processed away. The sequence of the disintegrin-like part of the Le-3 is identical to the A-chain of the heterodimeric disintegrin VLO5 from *Vipera lebetina obtusa* venom (Calvete et al 2003). The disintegrin-like domain of the Le-3 exists as a monomer in the heterodimeric disintegrin in *Vipera lebetina* venom (unpublished data). Dimeric disintegrins are widely distributed in *Echis* and *Vipera* venoms and probably also in the venoms of many other species of *Crotalidae* and *Viperidae* (Calvete et al 2005).

Thus the lebetase isoforms are P-I class proteins with a metalloproteinase domain only. The isoforms are synthesized from N-I class polynucleotides, comprising a signal peptide, a proenzyme and mature metalloproteinase domains in the deduced amino acid sequence, or N-II class polynucleotides, which additionally contain a disintegrin-like domain next to the proteinase domain. In the latter case the enzyme is synthesized in the venom gland as a P-II type proteinase (Fox and Serrano 2005), which is posttranslationally cleavaged to a mature (P-I class) proteinase. The results obtained suggest that even almost identical proteins may be synthesized in different ways. These results confirm the diversity of enzymes found within the same individual, while such diversity would be even greater between individuals within the same species. The content of lebetase in different venom batches is variable (Siigur et al 1998b). The ratio of the mature isoforms needs further investigation.

3.1.2. Factor X activator (VI)

The factor X activator from *Vipera lebetina* venom is one of the potent nonphysiological activators found among coagulant proteins from snake venoms. The VLFXA consists of the heavy chain (HC) and two light chains, LC1 and LC2. A metalloproteinase domain, a disintegrin-like domain and a cystein-rich domain are contributed to the mature protein by heavy chain whereas lectin-like domains by light chains. The heavy chain and the light chain LC1 are glycosylated.

3.1.2.1. Description of sequences

3.1.2.1.1 Heavy chain of VLFXA

The cDNA of the precursor of the heavy chain (2425 bp) of VLFXA includes a 5'-untranslated region, an open reading frame of 1839 bp and a 3'-untranslated region (Table 1). The open reading frame consists of a signal peptide region (18 amino acids), a proregion (170 amino acids), a metalloproteinase region (207 amino acids), a spacer region (18 amino acids), a disintegrin-like region (78 amino acids) and a cysteine-rich region (121 amino acids) (Fig. 7). Thus the ORF encodes a precursor protein of 612 amino acids comprising VLFXA HC of 419 amino acids with a calculated molecular mass 46,881 Da. The N-terminal protein sequence of the heavy chain of VLFXA determined by the protein sequencing is 100% identical with the sequence deduced from the cDNA. The N-terminal sequence of RVV-X (factor X activator from *Vipera russelli* venom) is five amino acids longer (Takeya et al 1992) and these additional amino acids described in the RVV-X sequence also exist in the precursor of VLFXA HC. It is not clear whether the N-terminus is processed differently or the studied

VLFXA HC has a truncated N-terminus. However, the N-terminal sequencing of the heavy chain of the VLFXA revealed two sequences with a single amino acid difference in their length. This means that either processing of VLFXA HC is heterogeneous with a shift of one amino acid residue or one amino acid is removed from about half of the molecules later on during the collection of the venom or purification. It has been shown previously that VLFXA preparations contain several isoforms (Siigur et al 2001). 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 (NXS/T) in the deduced sequence. However, it is still not clear if all these sites (Asn259, Asn353 and Asn373) are linked with carbohydrates (Fig. 7). In general, glycosylation has been thought to play a role in the stabilization of protein conformation and protection from proteolysis, as well as in cell-surface recognition phenomenon.

The deduced mature protein sequence refers to a strong similarity with other snake venom metalloproteinases, including proteins with different functions: factor X activators, prothrombin activators, hemorrhagic proteinases, and vascular apoptosis inducing proteinases. The VLFXA HC precursor is similar with P-III class proteinase precursors; having the highest similarity with *Vipera russelli* factor X activator heavy chain – 83% identity (Takeya et al 1992); slightly lower with ecarin – 72% identity (Nishida et al 1995), 72% with HR1a from *Trimeresurus flavoviridis* (Kishimoto and Takahashi 2002), 66% with berythractivase (Silva et al 2003), 65% with pro-catrocollastatin from *Crotalus atrox* (Zhou et al 1995), 63% with VAP1 (Masuda et al 2000). The VLFXA heavy chain has 42-30% identities with various ADAMs, being most similar with ADAM-28 (Cerretti et al 1999).

3.1.2.1.2. Light chains of VLFXA – LC1 and LC2

The cDNA encoding the light chain LC1 of VLFXA (709 bp) includes a 5'-untranslated region, an open reading frame of 438 bp and a 3'-untranslated region (Table 1). The open reading frame consists of 146 amino acids including 23 amino acids of propeptide and 123 amino acids of LC1. The N-terminal sequence of LC1 determined by the protein sequencing is 100% identical to the sequence deduced from the cDNA. The protein sequence of LC1 includes one potential *N*-glycosylation site at Asn47. The calculated molecular mass of the LC1 is 14,344 Da. The molecular masses of tryptic fragments of VLFXA LC1 confirm the protein sequence deduced from the cDNA.

The cDNA encoding the light chain LC2 (748 bp) includes a 5'-untranslated region, an open reading frame of 474 bp and a 3'-untranslated region (Table 1). The open reading frame consists of 158 amino acids including 23 amino acids of propeptide and 135 amino acids of LC2. The N-terminal sequence of LC2, as well as the inner sequence fragment determined by the protein sequencing is 100% identical to the sequence deduced from the cDNA. However, as in the case of heavy chain, the direct protein sequencing of the LC2 revealed two types of N-terminal sequences, which differ in one base pair in length. An explanation for this heterogeneity could be the same as the one above for the heavy chain. There are no potential *N*-glycosylation sites in the protein sequence of LC2. The calculated molecular mass is 15,830 Da. The molecular masses of tryptic fragments of VLFXA LC2 confirm the protein sequence deduced from the cDNA.

Comparison of the nucleotide sequence encoding the VLFXA light chain 1 revealed similarities with the cDNA encoding factor IX/X binding protein B chain from *Trimeresurus flavoviridis* venom (Matsuzaki et al 1996) and the cDNA encoding convulxin beta chain from *Crotalus durissus terrificus* venom (Leduc and Bon 1998). Comparison of the deduced protein sequence confirmed a similarity with other snake venom C-type lectins: 77% identity with RVV-X LC1 (Takeya et al 1992), 56% identity with the factor IX/X BP B-chain (Matsuzaki et al 1996), 55% identity with the convulxin beta chain (Leduc and Bon 1998). These proteins have different cellular functions in envenomation process (Tani et al 2002, Harrison et al 2003).

Comparison of the nucleotide sequence encoding VLFXA LC2 with other cDNA sequences revealed the highest similarity with the cDNA encoding VLFXA LC1, stejaggregin

from *Trimeresurus stejnegeri* (Xu et al 1999), agglucetin from *Deinagkistrodon acutus* (Wang et al 2003b). Recently the cDNA sequence of RVV-X LC2 has been deposited in the GenBank under the accession number DQ060414 (*Daboia russellii siamensis* C-type lectin-like protein subunit 1). The VLFXA LC2 identity with the latter one is 82%. The closest protein homologs to VLFXA LC2 are A(α)-chains of heterodimeric C-type lectins, such as antithrombin 1 A-chain from *Deinagkistrodon acutus* (56%), agglucetin-α2 subunit (56%) (Wang et al 2003b), alboaggregin A-2 (57%) (Kowalska et al 1998).

3.1.2.2. Characterization of the VLFXA

The VLFXA HC precursor has, like other snake venom metalloproteinases and matrix metalloproteinases, a "cysteine switch" motif PKMCGV in the prodomain (Grams et al 1993). The deduced amino acid sequence of VLFXA HC has a Zn-binding motif **H**ELG**H**NLGMY**H** in the catalytic domain followed by aspartic acid like in typical reprolysins (Fig. 7). The disintegrin-like domain contains ECD motif (or in some cases DCD) that is characteristic of many P-III type reprolysins (Nishida et al 1995, Kishimoto and Takahashi 2002, Silva et al 2003, Selistre de Araujo and Ownby 1995, Zhou et al 1995, Junqueira-de-Azevedo and Ho 2002, Masuda et al 2000) and also of ADAM metalloproteinases (Cerretti et al 1999).

The structure of VLFXA closely resembles that of the factor X activating enzyme from *Vipera russellii* venom (RVV-X) comprising three polypeptide chains bound by S-S bonds. The LC2-LC1 complex bears homology with heterodimeric C-type lectin-like proteins. LC2 is similar to A(α) chains of heterodimers and LC1 to B(β) chains which are linked by an interchain S-S bond. VLFXA LC2 includes eight half-cysteines and resembles flavocetin-A (Shin et al 2000) and convulxin (Leduc and Bon 1998) α-chains having cysteine at the C-terminus. This extra cysteine is probably involved in the interchain S-S bond formation connecting LC2 with the VLFXA heavy chain (HC-LC2-LC1). In the heavy chain of RVV-X, the Cys389 has been suggested to participate in the interchain S-S bond (Takeya et al 1992). In VLFXA, the Cys386 is the corresponding cysteine which might be connected with Cys135 in LC2. Based on analogy with P-III type metalloproteinases and C-type lectin-like proteins the hypothetical scheme of interchain disulfide bonding for VLFXA was proposed (Fig. 6).

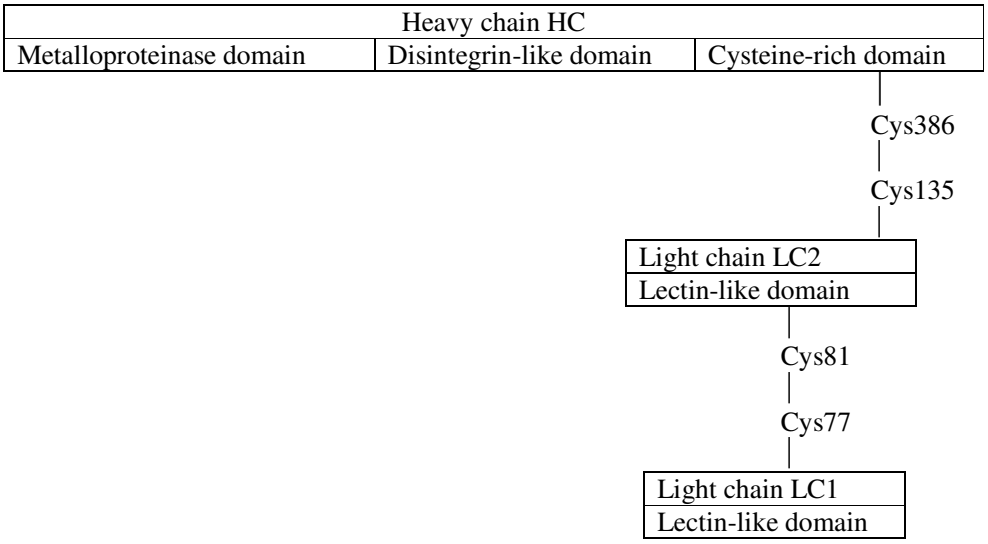

Figure 6. Hypothetical scheme of interchain disulfide bonds of VLFXA.

Light chains would serve as a regulatory region that determines the specificity of VLFXA by binding to the Gla-domain of the factor X, then the catalytic subunit (heavy chain) can cleave the particular bond in the heavy chain of the factor X releasing the active factor

Xa. The exact role of these various domains in the context of other domains in the overall enzymatic/biological activity of the protein is a question to be answered in the future.

We have showed that the heavy chain of VLFXA is synthesized from the N-III polynucleotide class and the heavy chain and light chains of VLFXA are synthesized from different genes. These results contradict the earlier proposal of a putative N-IV class of polynucleotide sequence for snake venom metalloproteinases. The latter has been speculated to encode information for a signal, a pro-, a proteinase, a disintegrin-like, a cysteine-rich and lectin-like domains, so that a protein could be translated as a six-domain precursor protein from one long transcript that is proteolytically processed to produce the P-IV class protein (Bjarnason and Fox 1994).

Thus the VLFXA is an example of a P-IV class protein which contains a proteinase domain, a disintegrin-like domain, a cysteine-rich domain and lectin-like domains that are synthesized from three different genes.

3.1.3. Endothelial cell apoptosis inducing metalloproteinase (VII)

An endothelial cell apoptosis inducing metalloproteinase (VLAIP) is a heterodimeric glycoprotein with a molecular mass about 106 kDa.

3.1.3.1. Description of sequences

The cDNAs of the precursors of monomers (VLAIP-A and VLAIP-B) include a 5'-untranslated region, an open reading frame of 1851 bp for VLAIP-A and of 1842 for VLAIP-B and a 3'-UTR (Table 1). The open reading frames consist of a signal peptide region (18 amino acids), a proregion (176 amino acids for VLAIP-A, 175 amino acids for VLAIP-B), a metalloproteinase region (205 amino acids for both VLAIP monomers), a spacer region (18 amino acids), a disintegrin-like region (78 amino acids) and a cysteine-rich region (121 amino acids for VLAIP-A and 120 amino acids for VLAIP-B) (Fig. 7). The ORF for VLAIP-A encodes a precursor protein of 616 amino acids comprising VLAIP-A of 422 amino acids with a calculated molecular mass of 46,840 Da. The ORF for VLAIP-B encodes a precursor protein of 614 amino acids comprising VLAIP-B of 421 amino acids with a calculated molecular mass of 47,020 Da. 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 the amino acid sequences deduced from the cDNA. There is one potential *N*-glycosylation site in VLAIP-A and four in VLAIP-B. However, it remains to be identified whether all these sites are linked with carbohydrates.

Comparison of the mature protein sequences revealed a strong similarity with those of other snake venom metalloproteinases, particularly with vascular apoptosis inducing proteinases: halysase from *Gloydius halys* venom (You et al 2003) - 76% identity with VLAIP-A, 75% with VLAIP-B; VAP1 from *Crotalus atrox* (Masuda et al 2000) - 75% identity with VLAIP-A, 74% with VLAIP-B; HV-1 from *Trimeresurus flavoviridis* (Masuda et al 1998, Masuda et al 2001) - 73 % identity with both VLAIP monomers. The other reprolysins with different specificities have also relatively high similarities with VLAIPs, eg the prothrombin activator berythractivase from *Bothrops erythromelas* (Silva et al 2003) and the factor X activator heavy chain VLFXA HC from *Vipera lebetina* - 69-70%. The highest identity is between VLAIP-A and VLAIP-B - 83%. VLAIP is also related to ADAM-28 (Howard et al 2000) – 42.9% identity with VLAIP-A and 43.2% with VLAIP-B.

3.1.3.2. Characterization of the enzyme

As the direct N-terminal sequencing of VLAIP resulted in no sequence, the amino terminus of the protein seemed to be blocked similarly to the sequences in many other snake venom metalloproteinases (Siigur et al 1998b). Both VLAIP precursors have a typical "cysteine switch" motif in their prodomains. The deduced amino acid sequences of a typical Zn-binding motif are **H**EMG**H**NLGME**HD** for VLAIP-A and **H**EMG**H**NLGMD**H**D for

VLAIP-B (Fig. 7). Based on the information about the conserved intrachain disulfide bond pattern for metalloproteinases containing six cysteine residues, the three S-S bonds may be formed within monomers leaving the cysteine in the position 369 free. That cysteine may act in the formation of an interchain disulfide bond between the two monomers of VLAIP. The cysteine 369 seems to be characteristic of vascular apoptosis inducing proteinases; many other snake venom metalloproteinases have other amino acids in this position. The disintegrin-like domain contains an ECD motif. As mentioned above, the ECD motif is characteristic of many P-III type reprolysins and also of ADAM metalloproteinases. The regions of the ECD motif are identical for both VLAIP monomers, and might be essential for binding to integrins. The exact molecular mechanisms, by which VLAIP exerts its effects on a cell (eg HUVEC) adhesion, as well as to induction of apoptosis, remain to be established.

Up to now several apoptosis inducing metalloproteinases have been isolated from snake venoms. These enzymes belong to different protein classes (P-I, P-III) and exert their apoptotic activity by ways, the mechanisms of which are not fully understood.

The P-I class metalloproteinase graminelysin-1 from *Trimeresurus gramineus* inhibits the adhesion of HUVECs to immobilized fibrinogen in a dose-dependent manner. The apoptosis-inducing activity probably results from the catalytic activity of graminelysin-1 on matrix proteins (Wu et al 2001). Another P-I class metalloproteinase – BaP1 from *Bothrops asper* – induces apoptosis via caspase-8 activation and cellular FLICE-inhibitory protein up-regulation, which is completely dependent on BaP1 enzymatic activity (Diaz et al 2003).

The homodimeric P-III class metalloproteinase VAP1 has been proposed to induce apoptosis of HUVECs caspase-independent way. The targets of a proteinase or a disintegrin domain of the VAP1 are not clear (Maruyama et al 2005). The apoptosis of endothelial cells induced by halysase (P-III class protein) is associated with the activation of caspase-3 and a decreased level of Bcl-X(L)/Bax. The contribution of the proteinase domain and the disintegrin-like domain of halysase to the induction of endothelial cell apoptosis is not fully understood (You et al 2003). The caspase-3 activation and the Bcl-X(L)/Bax ratio alterations mediated apoptosis is induced by one more P-III class protein – jararhagin from *Bothrops jararaca*. The endothelial cells specific apoptosis induction is associated with the enzymatic activity of the protein (Tanjoni et al 2005).

In conclusion, both the VLAIP monomers belong to P-III class proteins which contain proteinase domains, disintegrin-like domains and cysteine-rich domains, and which are synthesized from the N-III polynucleotide class. It is not clear how these different domains contribute to the vascular apoptosis inducing activity in the case of the VLAIP enzyme. Another yet unanswered question is the relevance of heterodimers of the molecule: is the enzymatic (apoptotic) activity dependent on the dimeric nature of the protein? Is the activity caused by the contribution of one monomer or both monomers? The cysteine 369 is presumably important for proteinases that induce vascular apoptosis. Further studies should be carried out to clarify these problems.

3.1.4. Comparison of sequences of metalloproteinases from *Vipera lebetina* venom

Table 1. The length of UTR and ORF sequences of metalloproteinases of the current study.

	5'-UTR	ORF	3'-UTR
VLAIP A	78 bp	1851 bp	288 bp
VLAIP B	78 bp	1842 bp	288 bp
Factor X activator HC	78 bp	1839 bp	508 bp
Factor X activator LC1	72 bp	438 bp	199 bp
Factor X activator LC2	78 bp	474 bp	196 bp
Lebetase isoform Le-3	89 bp	1437 bp	485 bp
Lebetase isoform Le-4	-	+609 bp	503 bp

Snake venom metalloproteinases, which have been characterized in the current work, are transcribed as precursors. The open reading frames of all of them (except that of the

lebetase clone Le-4 with a 5'-truncated end) consist of a signal peptide region, a proregion and a metalloproteinase region. All non-truncated sequences have a conserved "cysteine switch" motif PKMCGV in their prodomain, which is responsible for the activation of the corresponding enzyme (Fig. 7).

The lebetase clone Le-4 encodes the P-I class snake venom metalloproteinase. The lebetase clone Le-3 encodes the P-II class snake venom metalloproteinase, in which the metalloproteinase domain is followed by a disintegrin-like domain. The endothelial cell apoptosis inducing metalloproteinase (VLAIP) monomers (VLAIP-A and VLAIP-B) are synthesized as P-III class snake venom metalloproteinases comprising next to the metalloproteinase and disintegrin-like domains the cysteine-rich domains. The heavy chain of the factor X activator (VLFXA) is produced as a P-III class snake venom metalloproteinase as well.

All of the described metalloproteinases contain a Zn-binding motif **H**EXX**H**XXGXX**H** followed by aspartic acid (typical for reprolysins) in the catalytic domain and Met-turn CI/VM (typical for proteins of metzincin superfamily). The ECD motif in the disintegrin-like domains of VLAIP monomers and in the heavy chain of VLFXA is typical of P-III class reprolysins and might be essential for binding to integrins (Fig. 7).

VLAIP monomers and the heavy chain of VLFXA are glycosylated. There are three potential *N*-glycosylation sites (Asn-X-Ser or Asn-X-Thr, where X could be any amino acid except Pro) in the deduced protein sequence of the heavy chain of VLFXA, one potential *N*-glycosylation site in the VLAIP-A and four in the VLAIP-B sequences (Fig. 7).

```
                          1                                                  50
VLAIP A                   MMQVLLVTISLAVFPYQGSSIILESGNVNDYEVVYPQKVTAMPKGAVKQP
VLAIP B                   MMQVLLVTICLAVFPYQGSSIILESGNVNDYEVVYPQKITALPKGAIQQP
Factor X activator HC     MMQVLLVTISLAVFPYQGSSIILESGNVNDYEVVYPQKITALPEEAVQQP
Lebetase isoform Le-3     MIQVLLVTICLAVFPYQGSSKTLKSGNVNDYEVVNPQAVTGLPKGAVKQP
Lebetase isoform Le-4     --------------------------------------------------
                          *:*******:**********::*:**********:**::*::*::*::**
                          <Signal sequence >< prepropeptide

                          51                                                 100
VLAIP A                   EQKYEDAMQYEFKVKGEPVVLLLEKNKDLFSEDYSETHYSPDGREITTNP
VLAIP B                   EQKYEDAIKYEFKVNGKPVVLHLEKNKGLFSEDYSETHYTPDGREITINP
Factor X activator HC     EQKYEDTMQYEFEVNGEPVVLHLEKNKDLFSEDYSETRYSPDGRETTTKP
Lebetase isoform Le-3     EKKYEDTMQYEFEVNGEPVVLHLEKNRGLFSKDYSETHYSPDGREITTNP
lebetase isoform Le-4     --------------------------------------------------
                          *:****:::***:*:*:***::****::***:*****:*:*****:*::*

                          101                                                150
VLAIP A                   PVEDHCYYHGRIQNDADSSASISACNGLKGHFMLQGETYLIEPLKLPDSE
VLAIP B                   PVEDHCYYHGRIQNDADSTASISACNGLKGHFKLQGEMYLIEPLRIPDSE
Factor X activator HC     PVQDHCYYHGRIQNDAYSSASISACNGLKGHFKLQGETYLIEPLKIPDSE
Lebetase isoform Le-3     AVEDHCYYHGRIQNDADSTASISACNGLKGYFTLRGETYLIEPLKLPDSE
Lebetase isoform Le-4     --------------------------------------------------
                          :*:*************:*:***********:*:*:**:******::****

                          151                                                200
VLAIP A                   AHAVYKYENVEKEDEAPKMCGVTQTNWESDEPIKKASQLNLTPEQRRYLN
VLAIP B                   AHAIYKYENIEKEDEAPKMCGVTQTNWESDEPIK-ASQLNLTPEQRTYLK
Factor X activator HC     AHAVYKYENIEKEDEAPKMCGVTQTNWESDEPIKKASQLVATSAKRKFHK
Lebetase isoform Le-3     AHAVYKYENIEKEDEAPKMCGVTQTNWASDEPIKKASQLNLTPEQQRFEP
Lebetase isoform Le-4     -----------------------------SCRKKASQLNLTPEQQRFDP
                          ***:*****:*****************:**:::*:****::*:::::::::
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                    201                                                 250
VLAIP A             SPKYIKLVIVADYIMFLKYGRSLITIRTRIYEIVNILNVIYRVLNIYIAL
VLAIP B             SKKYVELVIVADYIMFWKYDRSLSTIRTRIYEIVNTLNVIYRFLNIYIAL
Factor X activator HC T--FIELVIVVDHRVVKKYDS--AATNTKIYEIVNTVNEIFIPLNIRLTL
Lebetase isoform Le-3 R--YIELVIVADHAMVTKYNGDLAAITTWVHQLVNNINGFYRDLNVHITL
Lebetase isoform Le-4 R--YIELVIVADHSMVTKYDGDLAAIRTWAHQLVNNIIVFYRDLNVHITL
                    :::::::****:*::::::*::::::::*:::::**:::::::::**::::*
                          metalloproteinase

                    251                                                 300
VLAIP A             LGLEIWNNGDKINVLPETKVTLDLFGKWRERDLLNRRKHDNAQLLTDINF
VLAIP B             VAVEIWSKGDLINVTSSAYDTLDSFGEWRERDLLNRKRHDNAQLLTGINF
Factor X activator HC IGVEFWCNRDLINVTSSADDTLDSFGEWRGSDLLNRKRHDNAQLFTDMKF
Lebetase isoform Le-3 SAVEVWTNGDLINVQPAASVTLNLFGEWRERDLLNRRMHDHAQLLTGIDL
Lebetase isoform Le-4 SAVEVWTNGDLINVQPAASVTLNLFGEWRERDLLNRRMHDHAQLLTAINL
                    :::*:*::::*:***::::::**::*****::*****::**:***:*::::

                    301                                                 350
VLAIP A             NGPTAGLGYVGSMCDPQYSAGIVQDHNKVNFLVALAMAHEMGHNLGMEHD
VLAIP B             NGPSAGRGFVGRMCQPKYSVGIVQDHSKIYLLVASAMAHEMGHNLGMDHD
Factor X activator HC DLSTLGITFLDGMCQAYRSVGIVQEHGNKNFKTAVIMAHELGHNLGMYHD
Lebetase isoform Le-3 DDNIIGLAYDDSMCDPRYSVGIVQDHSAIIRLVAVTMAHELGHNLGMNHD
Lebetase isoform Le-4 DDNTIGLAYNEGMCDPKYSVGIVQDHSAINRMVAATMAHEIGHNLGMDHD
                    :::::*::::::::**::::*:****:*::::::::*::****:******:**

                    351                                                 400
VLAIP A             EIHCTCGAKSCIMSGTLSCEASIRFSNCSREEHQKYLINKMPQCILNKPL
VLAIP B             RIDCTCGAKSCIMSGILRCETSYLFSDCSREEHRKYLINKMPQCILNKPL
Factor X activator HC RKNCICNDSSCIMSAVLSSQPSKLFSNCSNHDYRRYLTTYKPKCILNPPL
Lebetase isoform Le-3 GDQCNCGANGCVMSVVLIEQRSYQFSDCSKNKYQTYLTNRNPQCILNQPL
Lebetase isoform Le-4 GNQCNCGANGCVMSAVITQQRSYQFSDCSKNKYQTYLTNHNPQCILNQP-
                    :::*:*::::*:**::::::::*::::**:**::::::**::::*:****:*:
                                                                      ><

                    401                                                 450
VLAIP A             KTDIVSPAVCGNYLVELGEDCDCGSPRDCQNPCCNAATCKLTPGSQCADG
VLAIP B             KTDIVSPAVCGNYFVEVGEECDCGSPANCQDRCCDAATCKLRPGAQCGDG
Factor X activator HC RKDIASPPICGNEIWEEGEECDCGSPKDCQNPCCDAATCKLTPGAECGNG
Lebetase isoform Le-3 RTDTVSTPVSGNELLQ-----------NSGNPCCDPVTCQPRRGEHCVSG
Lebetase isoform Le-4 ----------------------------------------------------
                    ::*::*::::::**::::::**:******:::::::**:::**::::*::*::*
                    spacer          >< disintegrin-like

                    451                                                 500
VLAIP A             ECCDQCKFRRAGTVCRPANGECDVSDLCTGQSAECPTDQFQRNGQPCQNN
VLAIP B             VCCYQCKFRRAGTVCRPANGECDVSDLCTGQSAECPTDQFQRNGQPCQNN
Factor X activator HC LCCEKCKIKTAGTVCRRARDECDVPEHCTGQSAECPADGFHANGQPCQNN
Lebetase isoform Le-3 KCCRNCKFLRAGTVCKRA-VEGDDMDDYCTGISSDCPRNPYKD--------
Lebetase isoform Le-4 ----------------------------------------------------
                    :**::**:::*****::*::::*::::***:*::**:::::::********
                                                                    ><
```

```
                        501                                                 550
VLAIP A                 NGYCYSGTCPIMGKQCISLFGASATVAQDACFQFNSLGNEYGYCRKENGR
VLAIP B                 KGYCYNGTCPIMEKQCISLFGASATVAQDSCFQFNRRGNHYGYCRKENNT
Factor X activator HC   NGYCYNGDCPIMTKQCISLFGSRATVAEDSCFQENQKGSYYGYCRKENGR
Lebetase isoform Le-3   ------------------------------------------------
Lebetase isoform Le-4   ------------------------------------------------
                        :****:*:****:********::****:*:***:*::*::********::
                              cysteine-rich

                        551                                                 600
VLAIP A                 KIPCAPQDVKCGRLYCFDNLPEHKNPCQIYYTPSDENKGMVDPGTKCGDG
VLAIP B                 KIACAPEDVKCGRLYCLDNSSGHKNPCQIYYIPSDENKGMVDPGTKCGDG
Factor X activator HC   KIPCAPQDIKCGRLYCLDNSPGNKNPCKMHYRCRDQHKGMVEPGTKCEDG
Lebetase isoform Le-3   ------------------------------------------------
Lebetase isoform Le-4   ------------------------------------------------
                        **:***:*:*******:**::::****:::*:::*::****:*****:**

                        601          616
VLAIP A                 KACSSNRQCVDVNTAY
VLAIP B                 MVCS-NGKCVDVTIAY
Factor X activator HC   KVCNNKRQCVDVNTAY
Lebetase isoform Le-3   ----------------
Lebetase isoform Le-4   ----------------
                        ::*::::::****::**
```

Figure 7. Comparison of protein sequences of metalloproteinases of the current study. Identical amino acids at the same position are marked by "*", different amino acids by ":".The highlighted sequences are the following: the cysteine switch motif is in the blue box, the Zn-chelating motif is in the red box, the Met-turn is in the grey box, the XECD motif is in the lilac box; the potential N-glycosylation sites are yellow.

The amino acid sequence data of fibrinolytic, nonhemorrhagic and hemorrhagic metalloproteinases did not allow the identification of special amino acids responsible for fibrinolytic activity.

3.2. SERINE PROTEINASES FROM THE *VIPERA LEBETINA* VENOM (IV)

In addition to coagulant and anticoagulant venomous metalloproteinases, *Vipera lebetina* venom contains serine proteinases affecting the hemostatic system.

Among serine proteinases found in *Vipera lebetina* venom, two glycosylated serine fibrinogenases (Siigur et al 1991, Mähar et al 1987) and the factor V activating enzyme (Siigur et al 1998a) have been described. Despite having homologous N-terminal sequences, they possess different molecular masses, glycosylation levels, substrate specificity and fibrinogen degradation characteristics.

The serine proteinase degrading the Bβ-chain of fibrinogen without fibrinolysis - *Vipera lebetina* β-fibrinogenase – is a typical representative of arginine esterases without caseinolytic activity (Siigur et al 1991). The basic serine proteinase – a nonfibrinolytic α-fibrinogenase – degrades the Aα-chain of fibrinogen, which alters the latter's clottability by thrombin. It hydrolyses casein but not arginine esters (Mähar et al 1987). This enzyme is unique among the known serine proteinases.

The factor V activator from *Vipera lebetina* venom is similar to the activator from *Vipera russelli* venom. It supposedly cleaves the Arg1545-Ser1546 bond in the human factor V as shown by the hydrolysis of the human factor V peptide fragment corresponding to the cleavage region (AWYLR↓SNNG) and thus converts the factor V to its active form (factor Va) (Siigur et al 2001).

3.2.1. Factor V activator (II and III)

Vipera lebetina factor V activator (VLFVA) is the first factor V activating enzyme, cloned from the snake venom gland.

3.2.1.1. Description of sequences

The cDNA of the VLFVA (sequence VLP1; 1563 bp) includes a 5'-untranslated region, an open reading frame of 777 bp and a 3'-UTR (Table 2). The open reading frame consists of 259 amino acids, including a signal peptide (18 amino acids), an activation peptide (6 amino acids) and a mature enzyme coding region (235 amino acids) (Fig. 11). The calculated molecular mass for VLXVA is 25,881 Da; a minimal molecular mass detected by MALDI-TOF mass spectrometry was 28,400 Da. VLFVA is known to be a glycoprotein (Siigur et al 1998a) and this discrepancy is caused by the presence of carbohydrate moiety in the protein molecule. There is one glycosylation site in the sequence of VLFVA.

In addition to VLFVA sequence (VLP1), three additional homologous sequences (VLP2, VLP3 and VLP4) were obtained by screening the cDNA library. The VLP2 sequence is shorter than VLP1, comprising 1293 bp (1563 bp in the case of VLP1). The full-length VLP2 sequence includes a 5'-untranslated region of 171 bp (165 bp in the case of VLP1), a signal peptide (54 bp in both cases), an activation peptide (18 bp in both cases), a mature protein coding region of 708 bp (three nucleotides longer than in the case of VLP1) and a 3'-UTR of 342 bp (621 bp in the case of VLP1) (Table 2; Fig. 11). So the open reading frame of VLP2 encodes the protein sequence (260 amino acids), which is one amino acid longer than that of VLP1. The additional amino acid is located in the mature protein region. The calculated molecular mass for VLP2 is 26,185 Da. In comparison with VLP1, VLP2 has one more putative glycosylation site in its sequence.

Nucleotide sequences VLP3 and VLP4 encode for the open reading frames of 260 and 259 amino acids, respectively. All four homologous sequences (VLP1, VLP2, VLP3 and VLP4) have identical signal peptide (consisting of mainly hydrophobic amino acid residues) and activation peptide (consisting of mainly hydrophilic amino acid residues) sequences. Translated mature protein sequences of VLP3 and VLP4 represent combinations of the sequences VLP1 and VLP2. In VLP3 the amino acids 16-60 coincide with the VLFVA (VLP1) sequence, the amino acids 61-245 coincide with the VLP2 sequence. In VLP4 molecule the peptide fragments match with VLP1 and VLP2 in the opposite order (Fig. 8).

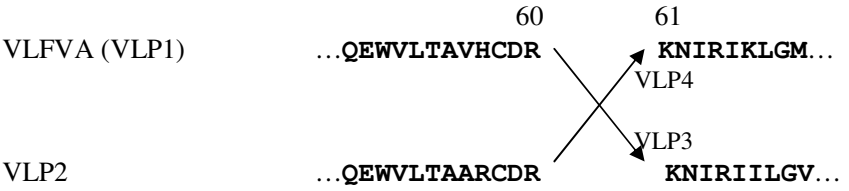

Figure 8. Schematic representation of homologous sequences VLFVA (VLP1), VLP2, VLP3 and VLP4.

The real existence of the "hybrid" DNAs in the *Vipera lebetina* cDNA library was verified by PCR using different pairs of primers for amplification of DNA fragments encoding amino acids 35-99. All primer pair combinations designed for verification of existence of these four homologous sequences gave a ~200 bp product in the PCR amplification. The sequencing of these products confirmed the presence of sequences of VLFVA (VLP1), VLP2, VLP3 and VLP4 in the *Vipera lebetina* cDNA library.

The nucleotide sequence of VLFVA (VLP1) has the highest similarity with the Tlg2b DNA from *Trimeresurus gramineus* venom (Deshimaru et al 1996) – 88%. The sequence identity in the proenzyme coding region is 100%. The signal peptide shares 98% identity with Tlg2b. The 3'- and 5'-UTRs of VLP1 have a 93 and 91% identity with Tlg2b, respectively.

The mature enzyme coding region is much more diversified than the noncoding regions, sharing only an 81% identity with Tlg2b. The identity is higher in the nucleotide sequences than in the translated protein sequence (88% versus 65%). This confirms that nucleotide substitutions in the protein-coding region tend to cause amino acid change. Comparison of the amino acid sequence of VLFVA with other snake venom serine proteinase sequences revealed the highest similarity with RVV-Vα and RVV-Vγ - 84% (Tokunaga et al 1988); with other snake venom serine proteinases the identity was lower (60-65%). VLFVA is also related to trypsin (41% identity). As for the VLFVA nucleotide sequence (VLP1), the sequence identity of VLP2 is highest with the DNA sequence from *Trimeresurus gramineus* venom (Deshimaru et al 1996), 88% identity with Tlg2a. Here also the identity is greatest in the proprotein coding region, reaching up to 100%. The signal peptide shares 98% identity with Tlg2a. The 3'- and 5'-UTRs of VLP2 have a 94 and 92% identity with Tlg2b, respectively. The mature enzyme coding region is also much more diversified than the noncoding regions, sharing an 88% identity with Tlg2a. The identity is higher in the nucleotide sequence when compared with the translated protein sequence (88% versus 78%).

In addition to protein sequence similarity of VLP2 with TLg2a (78%), it shares 60-70% identities with other snake venom serine proteinases and a 36% identity with trypsin. Among the proteins with known biological activity, the similarity is highest with the kinin-releasing and fibrinogen-clotting serine proteinase (KN-BJ) from the venom of *Bothrops jararaca* (Serrano et al 1998) – 69%.

3.2.1.2. Characterization of the enzymes

As a typical snake venom serine proteinase, the VLFVA (VLP1) contains a set of characteristic amino acid residues forming the catalytic triad (His57-Asp102-Ser195; Chymotrypsinogen numeration; Fig. 9). According to the first 3D structure of the snake venom serine proteinase – TSV-PA (the specific plasminogen activator from *Trimeresurus stejnegeri*), the active site cleft is located at the junction of the two six-stranded β-barrels (Parry et al 1998).

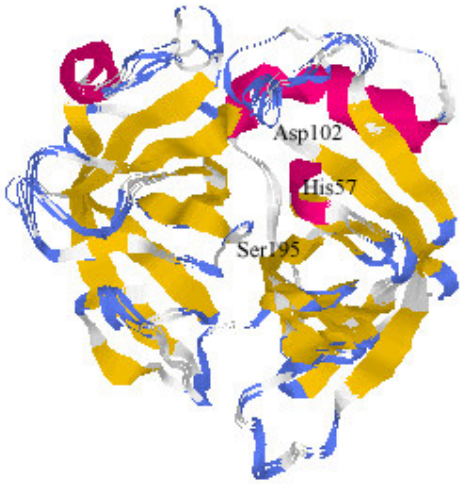

Figure 9. The model of VLFVA (VLP1) computed by SWISS-MODEL program (Schwede et al 2003, Guex and Peitsch 1997, Peitsch 1995).

12 strongly conserved cysteines enable to form 6 putative disulfide bonds as in other snake venom serine proteinases, but there may be considerable differences in the surface loops of various enzymes. The salt bridge, characteristic of the snake venom serine proteinases, can be formed between the N-terminal amino group of the first valine and the side chain of Asp194 preceding the catalytic Ser195. Conserved Asp189 at the bottom of a

"specificity pocket" near the active site allows the formation of a salt bridge with the basic residue of the scissile bond of the substrate whose cleavage may release the activated factor V in the case of VLFVA (VLP1). The Asp189 residue suggests that VLFVA (VLP1) has trypsin-type substrate specificity for basic amino acids at the P1 position (Fig. 11).

The sequence FPNG (95-98) seems to be characteristic only of factor V activators (RVV-V and VLFVA) and may be essential for factor V activation. The corresponding sequence KDDE in the plasminogen activator TSV-PA has been proven to be responsible for plasminogen activation (Zhang et al 1997). The authors explain this activity with the spatial proximity of the DDE-loop to the catalytic site. Amino acids which are characteristic only of factor V activators (VLFVA and RVV-V) and may contribute to the substrate specificity include Ser38, Arg135, Phe161, Trp167, Glu169, Asp178, Arg/His223 (a basic residue in this position; Chymotrypsinogen numeration).

Homologous sequences VLP2, VLP3 and VLP4 all have 12 conserved cysteines enabling formation of 6 disulfide bonds. In the case of VLP2 the catalytic triad characteristic of serine proteinases is mutated: histidine is replaced by arginine and serine by asparagine. In VLP3 the active site serine is replaced by asparagine, in VLP4 histidine – arginine replacement has taken place. Thus none of these homologous proteins contain the catalytic triad characteristic of serine proteinases.

The N-terminal part of the mature protein sequence of VLP2 (amino acids 16-54) matches well with the RVV-Vβ first 40 amino acids (Tokunaga et al 1988) sharing 95% identity. It was suggested (Tokunaga et al 1988) that RVV-Vβ is a serine proteinase but perhaps not factor V activator. The complete protein sequence of RVV-Vβ (as well as the nucleotide sequence) is still not available. The determined amino acid composition of RVV-Vβ significantly differed from those of RVV-Vα and RVV-Vγ (Tokunaga et al 1988).

The cDNA identified from the venom gland cDNA library of *Bitis gabonica* encodes a protein, in which the catalytic triad characteristic of serine proteinases is mutated similarly to VLP2: histidine is replaced by arginine and serine by asparagine (Francischetti et al 2004). Likewise the DNAs isolated from the venom gland cDNA libraries of *Trimeresurus gramineus* and *Trimeresurus flavoviridis, TLg2a* and *TLf2*, encoded for the proteins carrying mutations in the catalytic triad of serine proteinases: arginine residue instead of the active site histidine. The respective proteins from latter three species venoms have been neither isolated, nor expressed and their biological activity is still unknown (Deshimaru et al 1996, Francischetti et al 2004). As VLP2 protein has no catalytic triad characteristic of serine proteinases, despite of high homology of the primary structure, it is not a serine proteinase.

One possible explanation of generation of "hybrid" molecules VLP3 and VLP4 could be alternative (*trans*-)splicing. If to take into account the batroxobin gene organization (the only snake venom serine proteinase, whose gene organization has been published, similarity with VLP2 68%) (Itoh et al 1988) and to compare respective sequences of batroxobin with VLFVA and its homologs, it is evident that the braking point of the VLP3 and VLP4 sequences coincides with corresponding exon/intron borders of the batroxobin gene (Fig. 10).

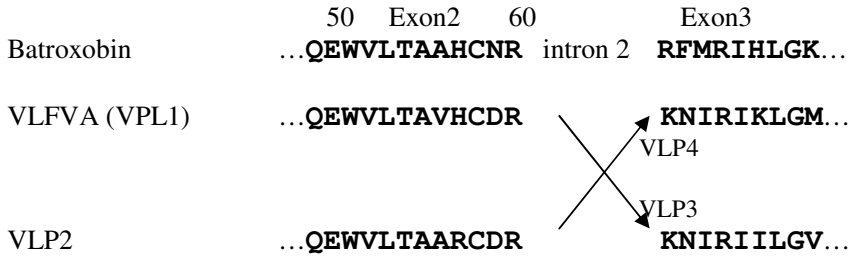

Figure 10. Scheme of putative formation of VLP3 and VLP4 by trans-splicing on the basis of the batroxobin gene organization (Itoh et al 1988).

Alternative splicing is well documented in the acetylcholinesterase gene of the snake *Bungarus fasciatus*, where different C-terminal exons are generated in the venom gland and muscle/liver (Cousin et al 1998). Alternative splicing event has been found in cobra venom, where ohanin, a protein inducing hypolocomotion and hyperalgesia in mice, is encoded by a single gene with five exons and four introns. Two types of mRNAs are generated by alternative splicing of exon 2 (Pung et al 2006). In Australian elapid the synthesis of two neurotoxin mRNA variants from one gene (Pt-sntx1) as a result of alternative splicing has been reported (Gong et al 2000).

Trans-splicing has been discovered in a few eukaryotic organisms like trypanosomes (Sutton and Boothroyd 1986), trematodes (Rajkovic et al 1990), the nematode worn (Krause and Hirsh 1987, Evans and Blumenthal 2000), several species of *Diptera* and *Lepidoptera* (Krauss and Dorn 2005). It was believed earlier that trypanosomes produce all of their mRNAs by *trans*-splicing, but by now the *cis*-spliced intron has been discovered, suggesting that these two splicing processes coexist in trypanosomes (Liang et al 2003). In nematodes only 10% of the mRNAs are synthesized by *trans*-splicing, which consequently would be involved in the post-transcriptional regulation of the particular protein synthesis (Tessier et al 1991). The *trans*-splicing was showed to occur in venom gland of scorpion *Buthus martensii* Karsch. It is suggested that *trans*-splicing may play a significant role in the diversification of scorpion venom peptides and may also play a critical role in the diversification of other animal venom peptides (Zeng et al 2005, Zhijian et al 2006). There are no references about *trans*-splicing in reptiles so far.

Another possible explanation of the generation of these “hybrid” molecules could be the genome alteration. The protein diversity can be achieved by mutations in the protein coding sequences, by exon shuffling, by gene duplication, by unequal crossing-over, by site-specific recombination etc. The focal hyper-mutation, block substitution and exon shuffling have been proposed to be the three main mechanisms leading to conotoxin diversity in *Conus striatus* (Pi et al 2006). The gene duplication as a base for the diversity of venom toxin has been proved in snakes as well as in venomous scorpions. Duplicated (multicopy) genes in different chromosomal locations have likely undergone different selective and evolutionary pressures and thus evolved into various highly homologous scorpion toxins (Cao et al 2005). In snakes the good examples are venom serine proteinase inhibitors that are members of the Kunitz/BPTI (bovine pancreatic trypsin inhibitor) family. These enzymes are believed to have been evolved by gene duplication and rapid diversification (Zupunski et al 2003). The gene duplication of an ancestral gene, followed by accelerated evolution, which led to five genes responsible for the synthesis of six isoforms of short-chain neurotoxin has also been described in Australian elapid *Pseudonaja textilis* (Gong et al 2000).

Exon shuffling could also lead to the creation of new genes. The idea of exon shuffling is that introns are as hot spots for genetic recombination. Introns affect the rate of homologous and nonhomologous recombination. However to make a new protein that contains the first part of one protein with the second part of another protein requires such a rare, and in frame event (Gilbert et al 1997).

So the further studies should be carried out to get more information about natural diversity and its origin within a single venom/animal.

The origin of these homologous sequences produced in *Vipera lebetina* venom remains still unknown. Wheater they are created by alternative (*trans*)-splicing or result from genome alteration needs to be clarified. It is also not clear whether all these molecules are expressed in the venom of the snake. The functions of the VLP2, VLP3 and VLP4 are also unknown.

Thus *Vipera lebetina* venom expresses a serine proteinase factor V activator. Besides, several homologous nucleotide sequences of VLFVA are transcribed in the venom gland, which carry mutations in their active sites. The origin of these transcripts is not clear, nor are the functions of their translation products. The real existence of corresponding proteins in the *Vipera lebetina* venom has not been proved either. The expression of these recombinant proteins is needed for their activity studies.

3.2.2. Anticoagulant serine fibrinogenases (V)

Vipera lebetina venom contains two anticoagulant serine proteinases: α-fibrinogenase (VLAF) and β-fibrinogenase (VLBF) (Siigur et al 1991, Samel et al 2002). VLAF has a molecular mass of 31.1 kDa and an isoelectric point >10. β-Fibrinogenase with the molecular mass of 42.2 kDa, is in contrast an acidic in its native form (pI<3) (Siigur et al 1991, Samel et al 2002).

3.2.2.1. Description of sequences

The cDNA of VLAF (1639 bp) includes a 5'-untranslated region, an open reading frame and a 3'-untranslated region (Table 2). The open reading frame consists of 258 amino acids, including a signal peptide (18 amino acids), an activation peptide (6 amino acids), and a mature enzyme coding region of 234 amino acids (Fig. 11). The VLBF sequence is shorter (1580 bp) than the sequence of the VLAF (1639 bp). The VLBF sequence includes a 5'-UTR (5 bp shorter than VLAF), a proenzyme coding region of the same length, a mature enzyme coding region 3 bp shorter than that of VLAF and a considerably shorter 3'-UTR (Table 2).

In conclusion, the open reading frames of both fibrinogenases (VLAF and VLBF) comprise 24 amino acids of a prepropeptide (18 amino acids of a signal peptide and 6 amino acids of an activation peptide) followed by 234 amino acid of a secreted enzyme sequence in the case of VLAF and 233 amino acids in the case of VLBF (Fig. 11).

The calculated molecular mass of VLAF is 25,910 Da and the isoelectric point is 10.6, which is in the good agreement with the pI of the native enzyme (>10). The molecular mass of the native enzyme detected by MALDI-TOF MS is 31.1 kDa (Samel et al 2002). The difference between calculated and determined molecular masses corresponds to approximately two carbohydrate moieties. There are three putative glycosylation sites in the VLAF sequence but not all of them need to be glycosylated.

The calculated molecular mass of VLBF is 25,580 Da, which is lower than that of the native enzyme (42.2 kDa) (Samel et al 2002). The difference is caused by heavy glycosylation of the native enzyme. Differences in the molecular masses point to 6 carbohydrate moieties VLBF should contain. VLBF has extremely high thermostability. It is known that glycosylation enhances the thermal stability of proteins (Wang et al 1996). The deduced protein sequence exhibits five putative *N*-glycosylation sites and one potential *O*-glycosylation site.

The highest similarity of VLAF was found with the cDNAs isolated from the cDNA libraries of *Trimeresurus gramineus* and *Trimeresurus flavoviridis*, TLg3, TLg2a, TLf3 (Deshimaru et al 1996) – identity of 85% from the beginning to the 3`-UTR. The identity in the 3`-UTRs is even higher (90%).

The highest similarity (87-88%) of VLBF was found also with the cDNAs of *Trimeresurus gramineus* and *Trimeresurus flavoviridis*, namely with TLf3, TLf2, TLg2a (Deshimaru et al 1996).

The similarity of protein sequences is highest between VLAF and VLBF themselves – 77%. The rate of similarity with other snake venom serine esterases covers the range of 60-73%. The nearest homologs of VLAF (after VLBF) are Bothrops protease A (AB031394) and TLg3 (Deshimaru et al 1996), 68 and 69% identity, respectively; the capillary permeability increasing enzyme, CPI-2 (Hahn et al 1998) and brevinase, a fibrin(ogen)olytic enzyme from *Agkistrodon blomhoffii brevicaudus* venom (Lee and Park 2000) share 67% identity with VLAF. VLBF shows the highest similarity (after VLAF) with brevinase – 73%. Brevinase and CPI-2 are typical arginine esterases devoid of caseinolytic activity.

3.2.2.2. Characterization of the enzymes

Like other snake venom serine proteinases, both the VLAF and VLBF contain a typical catalytic triad (His57-Asp102-Ser195; Chymotrypsinogen numeration) surrounded by

highly conserved regions. Both enzymes have twelve cysteine residues in strongly conserved positions enabling formation of six disulfide bonds.

Wang (2001) has proposed subtype-specific regions of venom proteinases (residues 82-99, 192-193, and 217-219; Chymotrypsinogen numeration). These regions are the known substrate-binding subsites. According to these amino acid sequences, VLAF and VLBF belong to the KN-subgroup (kininogenases) of serine proteinases although neither enzyme exhibited detectable kinin-releasing activity (Siigur et al 1991, Samel et al 2002).

As in almost all of the active serine proteinases, the particular N-terminal residue (Val16 in VLAF and VLBF) forms an internal salt bridge between its amino group and the side chain carboxyl group of Asp194 (Wang et al 1985). The conserved Asp189, which is characteristic to trypsin-like serine proteinases (Janin and Chothia 1990), is in the case of VLAF and VLBF replaced by Gly189, that may lead to the possible lack of the trypsin-type substrate specificity for basic amino acids at the P1 position (Fig. 11). This replacement deprives trypsin of arginine specificity and turns it into a nonspecific proteolytic enzyme (Czapinska and Otlewski 1999) like it has happened with VLAF. At the same time, VLBF behaves like an arginine esterase although the activity towards arginine esters is remarkably lower than that of trypsin (Siigur et al 1991). In this case perhaps some other, yet unknown, factors contribute to the redesign of the S1 pocket. The feature distinguishing VLAF from VLBF is the sequence – RRR – (amino acids 185-187) in the vicinity of the putative S1 pocket that may cause steric and electrostatic resistance to the positively charged substrates. However, VLAF appears to be a unique enzyme since there is currently no information about anticoagulant α-fibrinogenases with similar structure, which degrade the Aα-chain of fibrinogen only and lack the esterolytic activity.

Thus two anticoagulant serine proteinases, α-fibrinogenase (VLAF) and β-fibrinogenase (VLBF) share common features with other snake venom serine proteinases. Still the replacement of a conserved Asp189 by Gly189 may eventually cause alterations in substrate specificity.

3.2.3. Comparison of sequences of serine proteinases from *Vipera lebetina* venom

Table 2. The length of UTR and ORF sequences of serine proteinases of the current study.

	5'-UTR	ORF	3'-UTR
Factor V activator (VLP1)	165 bp	777 bp	621 bp
Factor V activator's homolog VLP2	171 bp	780 bp	342 bp
α-fibrinogenase (VLAF)	185 bp	774 bp	680 bp
β-fibrinogenase (VLBF)	180 bp	771 bp	629 bp

Snake venom serine proteinases and some homologous sequences of the current work are transcribed as zymogens. The open reading frames of all of them consist of a prepropeptide region and a mature enzyme (protein) region. The prepropeptide region begins with a signal sequence which is followed by an activation peptide region (Fig. 11).

All of the described serine proteinases (factor V activator – VLFVA (VLP1); α-fibrinogenase – VLAF; β-fibrinogenase – VLBF) have a characteristic set of amino acid residues, forming the catalytic triad (His57-Asp102-Ser195). All of the factor V activator homologs, which are characterized (VLP2, VLP3 and VLP4), lack the catalytic triad characteristic of serine proteinases. Nevertheless, all analyzed sequences have a residue Ser214, which appears to be conserved in chymotrypsin-like proteinases and is essential for substrate recognition (Fig. 11). All of described sequences have Asp194, which enables the salt bridge formation with the first valine, present in all of these sequences as well. This salt bridge is believed to stabilize the structure of the active site. The conserved Asp189 (also found in VLFVA and its homologs), which is characteristic of trypsin-like serine proteinases, has been replaced by Gly189 in VLAF and VLBF, probably leading to alterations in substrate specificity (Fig. 11).

All of the serine proteinases of the present work are glycosylated. There is one *N*-glycosylation site in the deduced protein sequence of VLFVA (VLP1), three putative *N*-glycosylation sites in VLAF, five putative *N*-glycosylation sites and one potential *O*-glycosylation site in VLBF sequence. The VLFVA homologs could also be glycosylated – there are two putative *N*-glycosylation sites in the deduced sequence of VLP2 (Fig. 11).

```
                                          16 20        30         40
β-fibrinogenase (VLBF)   MVLIRVLANLLLLQLSYAQKSSELVVGGDECNINEHRSLVFLYNSS---F
α-fibrinogenase (VLAF)   MVLIRVLANLVMLHLSYGEKSSELVIGGRPCNINQHRSLALLYNSS--GF
Factor V activator (VLP1)MVLIRVLANLLVLQLSYAQKSSELVVGGDECDINEHPFLVALYTSSSSTV
VLP1 homolog (VLP2)      MVLIRVLANLLVLQLSYAQKSSELVIGGDECNINEHPFPVALHTARSKRF
                         **********::*:***::******:**::*:**:*:::::*::::::::
                         <signal sequence ><actp><         mature protein
                         <     prepropeptide    ><         mature protein

                                  50         60        70         80         90
β-fibrinogenase (VLBF)   GCGGTLINQQWVLSAAHCDMENVQIYLGLHNLRLRNQDEQIRVAEEKFFC
α-fibrinogenase (VLAF)   LCGGTLINQQWVLSAAHCDMENMQIYLGLHNFSLPNMDQKRRVAEEKFFC
Factor V activator (VLP1)HCAGTLINQEWVLTAVHCDRKNIRIKLGMHSKNIRNEDEQIRVPRRKFFC
VLP1 homolog (VLP2)      YCAGTLINQEWVLTAARCDRKNIRIILGVHSKNVPNEDQQIRVPKEKFFC
                         :*:******:***:*::**::*::*:**:*::::::*:*:::**::****

                                       110         120               140
β-fibrinogenase (VLBF)   LSNKSYTKWDKDIMLIRLNSSVTYNTHIAPLSLPSSPPRVGSVCRIMGWG
α-fibrinogenase (VLAF)   LSNKSYTKWDKDIMLIKLNRRVKTSTHIAPLSLPSNPPRLRSVCRIMGWG
Factor V activator (VLP1)LNTKFPNGKDKDIMLIRLRRPVKNSAHIAPISLPSSPSSPRSRCRIMGWG
VLP1 homolog (VLP2)      LSSKTYTRWDKDIMLIRLKKPVNDSTHIVPLSLPSSPPSVGSVCRIMGWG
                         *::*::::::*******:*:::*::::::**:*:****:*::::*:*******

                                       160       170        180
β-fibrinogenase (VLBF)   AITSPNETFPNVPHCANINILRYSVCRAAYRGLPAQSRTLCAGILQGGIG
α-fibrinogenase (VLAF)   SITSPRETLPYVPHCANIMILRYWVCRAIYGSLPAKSRTLCAGVPRRRIG
Factor V activator (VLP1)KISTTEETYPDVPHCAKIFIVKHAWCEALYPWVPADSRTLCAGILQGGKD
VLP1 homolog (VLP2)      TITTTKVTYPDVPHCANINMFDYSVCRKVYRKLPEKSRTLCAGILQGGID
                         :*::::::*:*:*****:*::::::::*:::*:::*::*******::::::

                         190     200   210        220         230        240
β-fibrinogenase (VLBF)   SCMGDSGGPLICNGEIQGIVSWGDDICAQPHKPVHYTKVFDYSDWIQSII
α-fibrinogenase (VLAF)   SCLGDSGGPLICNGQIQGIASWGSDPCVNHGAPGVYTKVFDYNDWIQSII
Factor V activator (VLP1)TCEGDSGGPLICNGQIQGIVSGGSDPCGQRLKPAVYTKVFDYTDWIQSII
VLP1 homolog (VLP2)      SCKVDNGGPLICNGQIQGIVSWGGHPCAQPHKPALYTNVFDYTDWIQSII
                         :*::*:********:****:*:*:::*::::::*::**:****:*******

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β-fibrinogenase (VLBF)   AGNTTATCPL
α-fibrinogenase (VLAF)   AGNTAATCPP
Factor V activator (VLP1)AGNTTATCP-
VLP1 homolog (VLP2)      AGNITATCPP
                         ***::****:
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Figure 11. Comparison of protein sequences of serine proteinases of the current study. Identical amino acids at the same position are marked by "*", different amino acids by ":". The amino acids of the catalytic triad are indicated in red; the potential N-glycosylation sites are yellow, the conserved Ser214 are blue, Asp/Gly189 is in green, salt-bridge forming Val-Asp are in grey. Chymotrypsinogen numeration has been used. "Actp" denotes an activation peptide region.

4. CONCLUSIONS

The main results of the current study are the following:

1. Three metalloproteinases and three serine proteinases have been cloned from *Vipera lebetina* venom gland and their nucleotide sequences have been characterized.

2. We showed that the venom gland of *Vipera lebetina* synthesises proteins of each of the four classes of snake venom metalloproteinases (classes P-I – P-IV) including a heterodimeric protein (an endothelial cell apoptosis inducing metalloproteinase – VLAIP) whose monomers belong to the P-III class proteins.

3. The molecular characterization of a particular metalloproteinase lebetase suggests that even almost identical proteins may be synthesized in different ways – in the forms of P-I and P-II proteinases that in the latter case is posttranslationally cleaved to the mature P-I class protein.

4. The characterization of the factor X activator (VLFXA) resulted in revision of the synthesis scheme of the snake venom metalloproteinases classes. We demonstrated that the heavy chain and the light chains of VLFXA are synthesized from three different genes. It was speculated earlier that P-IV class proteinases are encoded by N-IV class of polynucleotides and are translated as six-domain precursor proteins from one transcript that is proteolytically processed to P-IV class protein.

5. The cloning of a coagulant snake venom serine proteinase – factor V activator (VLFVA) revealed three other homologous nucleotide sequences of VLFVA with mutations in their active sites.

6. Two anticoagulant fibrinogenolytic serine proteinases were cloned and sequenced. Based on the substrate specificity the α-fibrinogenase (VLAF) is unique among known serine proteinases and the β-fibrinogenase (VLBF) is a thermostable highly glycosylated arginine esterase.

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ABBREVIATIONS

ADAM	–	A Disintegrin And Metalloproteinase
BAEE	–	benzoylarginine ester
CNBr	–	cyanogen bromide
CRISP	–	cysteine-rich secretory protein
DFP	–	diisopropylfluorophosphate
DTT	–	dithiothreitol
EDTA	–	ethylenediaminetetraacetic acid
EGFR	–	epidermal growth factor receptor
EGTA	–	Ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetic Acid
FPA	–	fibrinopeptide A
FPB	–	fibrinopeptide B
HC	–	heavy chain
HUVEC	–	human umbilical vein endothelial cells
LC	–	light chain
LC-MS/MS	–	liquid chromatography-electrospray ionization tandem mass spectrometry
MALDI-TOF MS	–	matrix-assisted laser desorptsion ionization time of flight mass spectrometry
MW	–	molecular weight
ORF	–	open reading frame
PCR	–	polymerase chain reaction
PL	–	phospholipids
PLA_2	–	phospholipase A_2
PMSF	–	phenylmethylsulfonyl fluoride
PZP	–	pregnancy zone protein
SDS-PAGE	–	sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis
TAME	–	tosylarginine metyl ester
UTR	–	untranslated region
VLAF	–	*Vipera lebetina* α-fibrinogenase
VLAIP	–	*Vipera lebetina* endothelial cell apoptosis inducing metalloproteinase
VLBF	–	*Vipera lebetina* β-fibrinogenase
VLFXA	–	*Vipera lebetina* factor X activator
VLFVA	–	*Vipera lebetina* factor V activator

ABSTRACT

Snake venoms are rich in biologically active components, including proteins, which can be used for studying the basic mechanisms of hemostasis and envenomation to enable us to develop better diagnostic and therapeutic agents for clinical use. The information deriving from the snake venom protein analysis can be used for snake evolution estimation/reconstruction. The information, both at the structural and gene level, is needed for a better understanding of action mechanisms of venomous proteins. In the current work several snake venom metalloproteinases and serine proteinases affecting blood coagulation and hemostasis have been characterized.

We have shown that *Vipera lebetina* venom contains all four classes of snake venom metalloproteinases.

Among them, lebetase is the first fibrinolytic enzyme ever sequenced from *Viperidae* venom gland. The lebetase isoforms belong to the P-I class proteins and have a metalloproteinase domain only. The isoforms are synthesized from N-I class polynucleotides, comprising a signal peptide, a 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 the venom gland as a P-II class proteinase, 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 proteinase. The disintegrin-like part of the lebetase clone Le-3 exists as a monomer in the heterodimeric disintegrin in the *Vipera lebetina* venom. These results suggest that even almost identical proteins may be synthesized in different ways.

Our study of the factor X activator (VLFXA) resulted in a revision of the synthesis scheme of metalloproteinases classes. VLFXA is a glycoprotein composed of a heavy chain and two light chains linked by disulfide bonds. We demonstrated that the heavy chain of VLFXA is synthesized from the N-III polynucleotide class and the heavy chain of VLFXA and light chains of VLFXA are synthesized from different genes. These results contradict the earlier proposal of a putative N-IV class of a polynucleotide sequence for snake venom metalloproteinases, which has to be speculated to encode information for signal, pro-, proteinase, disintegrin-like, cysteine-rich and lectin-like domains. Thus the VLFXA is an example of a P-IV class protein that contains a proteinase domain, a disintegrin-like domain, a cysteine-rich domain and lectin-like domains that are synthesized from three different genes.

The next enzyme we cloned, an endothelial cell apoptosis inducing metalloproteinase (VLAIP), is a heterodimeric glycoprotein. Both VLAIP monomers are examples of P-III class proteins that contain metalloproteinase domains, disintegrin-like domains and cysteine-rich domains that are synthesized from the N-III polynucleotide class. The cysteine 369 may be important for proteinases inducing vascular apoptosis, as many other snake venom metalloproteinases have other amino acids in this position.

Among serine proteinases, *V. lebetina* factor V activator (VLFVA) was the first cloned factor V activating enzyme from snake venom. As a typical snake venom serine proteinase, the VLFVA (VLP1) has a characteristic set of amino acid residues, forming the catalytic triad (His57-Asp102-Ser195); strongly conserved cysteines enabling formation of disulfide bonds as in other snake venom serine proteinases; conserved residues Asp194 (involved in the salt bridge formation with the first valine), Asp189 (involved in substrate recognition). However, the sequence FPNG (95-98) is apparently characteristic only of factor V activators and may be essential for factor V activation.

Besides VLFVA (VLP1), several homologous sequences of VLFVA are transcribed in the venom gland with mutations in their active site. The origin of these transcripts is not clear, neither are the functions of translated putative proteins.

We also described two anticoagulant serine proteinases: α-fibrinogenase (VLAF) and β-fibrinogenase (VLBF). VLAF is unique among known serine proteinases for its substrate specificity. These enzymes share common features with other snake venom serine proteinases (catalytic triad, cysteine bond pattern), except for the replacement of a conserved Asp189 by Gly189, which eventually may cause alterations in substrate specificity.

KOKKUVÕTE

Maomürkides on hulgaliselt bioloogiliselt aktiivseid ühendeid, mida on võimalik kasutada hemostaasi ja mürgistuse toimemehhanismide uurimiseks, eesmärgiga töötada välja paremaid diagnostilisi vahendeid ja ravimeid. Maomürkide valkude analüüsimisel saadud informatsiooni saab kasutada ka madude evolutsiooni hindamiseks/rekonstrueerimiseks. Maomürkide valkude toimemehhanismi paremaks mõistmiseks on vaja informatsiooni nii geeni kui ka struktuuri tasemel. Käesolevas töös uuriti *Vipera lebetina* mürgi metalloproteinaase ja seriinproteinaase, mis mõjutavad hemostaasi.

Me näitasime, et *Vipera lebetina* mürk sisaldab kõiki nelja klassi kuuluvaid maomürgi metalloproteinaase.

Lebetaas on esimene *Viperidae* (rästiklaste) mürginäärmest sekveneeritud fibrinolüütiline ensüüm. Lebetaasi isovormid on metalloproteinaaside P-I klassi valgud, mis sisaldavad ainult metalloproteinaasi domääni. Isovormid sünteesitakse kas N-I klassi polünukleotiidjärjestuselt ning nende transleeritud järjestus koosneb signaalpeptiidist, pro- ja metalloproteinaasi domäänist või N-II klassi polünukleotiidjärjestuselt, mis lisaks sisaldab proteinaasi domääni järgselt disintegriinisarnast domääni. Viimasel juhul, lähtudes maomürkide metalloproteinaaside modifitseeritud klassifikatsiooniskeemist (Fox and Serrano 2005), sünteesitakse ensüüm mürginäärmes P-II klassi proteinaasina, millest posttranslatsiooniliselt (disintegriinisarnase domääni eemaldamise tulemusena) saadakse P-I klassi proteinaas. Lebetaasi klooni Le-3 disintegriinisarnane osa esineb monomeerina heterodimeerses disintegriinis. Saadud tulemused näitavad, et isegi peaaegu identseid valke võidakse sünteesida erineval viisil.

Meie teedrajav töö faktor X aktivaatori (VLFXA) kirjeldamisel tõi endaga kaasa maomürkide metalloproteinaaside klasside sünteesiskeemi ümbervaatamise. VLFXA on glükoproteiin, mis koosneb raskest ahelast ja kahest disulfiidsildadega ühendatud kergest ahelast. Meie töös on näidatud, et VLFXA raske ahel sünteesitakse N-III klassi polünukleotiidjärjestuselt ning VLFXA raske ahel ja kerged ahelad sünteesitakse erinevatelt geenidelt. Need tulemused lükkavad ümber varasema oletuse maomürgi metalloproteinaaside N-IV klassi polünukleotiidjärjestuse olemasolu kohta, mis arvati kodeerivat signaal-, pro-, proteinaasi, disintegriinisarnast, tsüsteiinirikast ja lektiinisarnast domääni. Kokkuvõttes on VLFXA näide P-IV klassi valgust, mis sisaldab proteinaasi domääni, disintegriinisarnast domääni, tsüsteiinirikast domääni ja lektiinisarnast domääni, mis sünteesitakse kolmelt erinevalt geenilt.

Endoteelirakkude apoptoosi põhjustav metalloproteinaas (VLAIP) on heterodimeerne glükoproteiin. Mõlemad VLAIPi monomeerid kujutavad endast P-III klassi valke, mis sisaldavad metalloproteinaasi domääne, disintegriinisarnaseid domääne ja tsüsteiinirikkaid domääne, mis sünteesitakse N-III klassi polünukleotiidjärjestustelt. Näib, et tsüsteiin 369 on iseloomulik vaskulaarset apoptoosi põhjustavatele proteinaasidele, sest paljudel teistel maomürgi metalloproteinaasidel on selles positsioonis teistsugused aminohapped.

Seriinproteinaaside hulgas oli *Vipera lebetina* faktor V aktivaator (VLFVA) esimene kloonitud faktor V aktiveeriv ensüüm maomürgist. Tüüpilisele maomürgi seriinproteinaasile iseloomulikult moodustavad VLFVA (VLP1) korral katalüütilise triaadi His57-Asn102-Ser195; konserveerunud tsüsteiinid võimaldavad disulfiisidemete moodustumist sarnaselt teistele maomürgi seriinproteinaasidele; konserveerunud on Asp194 (osaleb soolasilla moodustamises esimese valiiniga) ja Asp189 (osaleb substraadi äratundmises). Ent järjestus FPNG (95-98) näib olevat iseloomulik ainult faktor V aktivaatoritele ja seega võib osutuda oluliseks faktor V aktiveerimisel. Lisaks VLFVA (VLP1) järjestusele transkribeeritakse mürginäärmes mitmeid VLFVA järjestusega homoloogseid, muteerunud aktiivsaidiga järjestusi.

Vipera lebetina mürk sisaldab ka kahte antikoagulantset seriinproteinaasi: α-fibrinogenaasi (VLAF) ja β-fibrinogenaasi (VLBF). VLAF on unikaalne seni tuntud seriinproteinaaside hulgas oma substraadispetsiifilisuse poolest. Nende ensüümide sarnasused teiste maomürgi seriinproteinaasidega hõlmavad katalüütilist triaadi ja disulfiidsidemete mustrit. Erinevus on konserveerunud Asp189 asendus Gly189 vastu, mis ilmselt võib põhjustada muutusi substraadispetsiifilisuses.

CURRICULUM VITAE

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12.1985 – 10.1989	Estonian Forest Institute; chief research assistant
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5. Supervised dissertations (co-supervisor)

Ingri Sumeri "Polymorphism of Nuclear Short Tandem Repeats in an Estonian Population Sample" (Tallinn Technical University, MSc), 1999.

Eveli Raudvere "Geneetiliselt muundatud toiduainete analüüs polümeraasi ahelreaktsiooni meetodiga" (Detecting genetically modified food products by using polymerase chain reaction) (Tallinn Technical University, BSc), 2001.

Kärri Jürisson "Hemokromatoos ja selle diagnoosimine molekulaarsel tasemel" (Hemochromatosis and its molecular diagnosis) (Tallinn Technical University, BSc), 2002.

Kadri Lagemaa "Lühikeste DNA kordusjärjestuste analüüs eestlaste populatsioonis PowerPlex 16 abil" (The analysis of short tandem repeats (STRs) in an Estonian population sample using the PowerPlex™ 16 kit) (Tallinn Technical University, BSc), 2002.

Esther Masing "Mikrosatelliitsete markerite analüüs ja geneetiline mitmekesisus Soome pruunkaru näitel" (Finnish brown bear: analysis of microsatellite markers and genetic diversity) (Tallinn Technical University, BSc), 2002.

Tiiu Roovere „Y kromosoomi STR lookuste alleelide jaotus Eesti populatsiooni näitel" (The allelic distribution of the Y chromosomal STR loci in an Estonian population sample) (Tallinn Technical University, BSc), 2002.

Kadri Lagemaa „Koerte eristamine nende geneetilise varieeruvuse alusel" (Differentiating between dogs on the bases of their genetic variation) (Tallinn Technical University, MSc), 2005.

Esther Masing „Eesti ja Soome pruunkaru populatsioonide geneetiline analüüs" (Genetic analysis of the Estonian and Finnish brown bear populations) (Tallinn Technical University, MSc), 2005.

6. Research experience

Protein structure and functions, genetic analysis.
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Alates 05.1999	Kohtuekspertiisi ja Kriminalistika Keskus; ekspert, 12.1999.a. alates DNA labori peaekspert, 07.2002.a. alates keemia- ja bioloogiaosakonna juhataja

5. Kaitstud lõputööd (kaasjuhendaja)

Ingri Sumeri "Polymorphism of Nuclear Short Tandem Repeats in an Estonian Population Sample" (Tuumsete lühikeste kordusjärjestuste polümorfism eestlaste polulatsioonis) (Tallinna Tehnikaülikool, magistritöö), 1999.

Eveli Raudvere "Geneetiliselt muundatud toiduainete analüüs polümeraasi ahelreaktsiooni meetodiga" (Tallinna Tehnikaülikool, bakalaureusetöö), 2001.

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Esther Masing "Mikrosatelliitsete markerite analüüs ja geneetiline mitmekesisus Soome pruunkaru näitel" (Tallinna Tehnikaülikool, bakalaureusetöö), 2002.

Tiiu Roovere „Y kromosoomi STR lookuste alleelide jaotus Eesti populatsiooni näitel" (Tallinna Tehnikaülikool, bakalaureusetöö), 2002.

Kadri Lagemaa „Koerte eristamine nende geneetilise varieeruvuse alusel" (Tallinna Tehnikaülikool, magistritöö), 2005.

Esther Masing „Eesti ja Soome pruunkaru populatsioonide geneetiline analüüs" (Tallinna Tehnikaülikool, magistritöö), 2005.

6. Teadustöö põhisuunad

Valkude struktuur ja funktsioonid, nende geenianalüüs.
Genoomiuuringud.
Tegevusuunad kajastuvad täpsemalt artikites ja juhendamisel kaitstud lõputöödes.

LIST OF PUBLICATIONS

1. Truve, E., **Aaspõllu, A.**, Honkanen, J., Puska, R., Mehto, M., Hassi, A., Teeri, T.H., Kelve, M., Seppänen, P., Saarma, M. (1993) Transgenic potato plants expressing mammalian 2'-5' oligoadenylate synthetase are protected from potato virus X infection under field conditions. Biotechnology (N Y). 11:1048-1052.

2. Truve, E., Kelve, M., **Aaspõllu, A.**, Kuusksalu, A., Seppänen, P., Saarma, M. (1994) Principles and background for the construction of transgenic plants displaying multiple virus resistance. Arch Virol Suppl. 9:41-50.

3. Truve, E., Kelve, M., **Aaspõllu, A.**, Schröder, H.C., Müller, W.E. (1994) Homologies between different forms of 2-5A synthetases. Prog Mol Subcell Biol. 14:139-149.

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11. Siigur, E., **Aaspõllu, A**., Siigur, J. (2001) Sequence diversity of *Vipera lebetina* snake venom gland serine proteinase homologs – result of alternative-splicing or genome alteration. Gene 263, 199-203.

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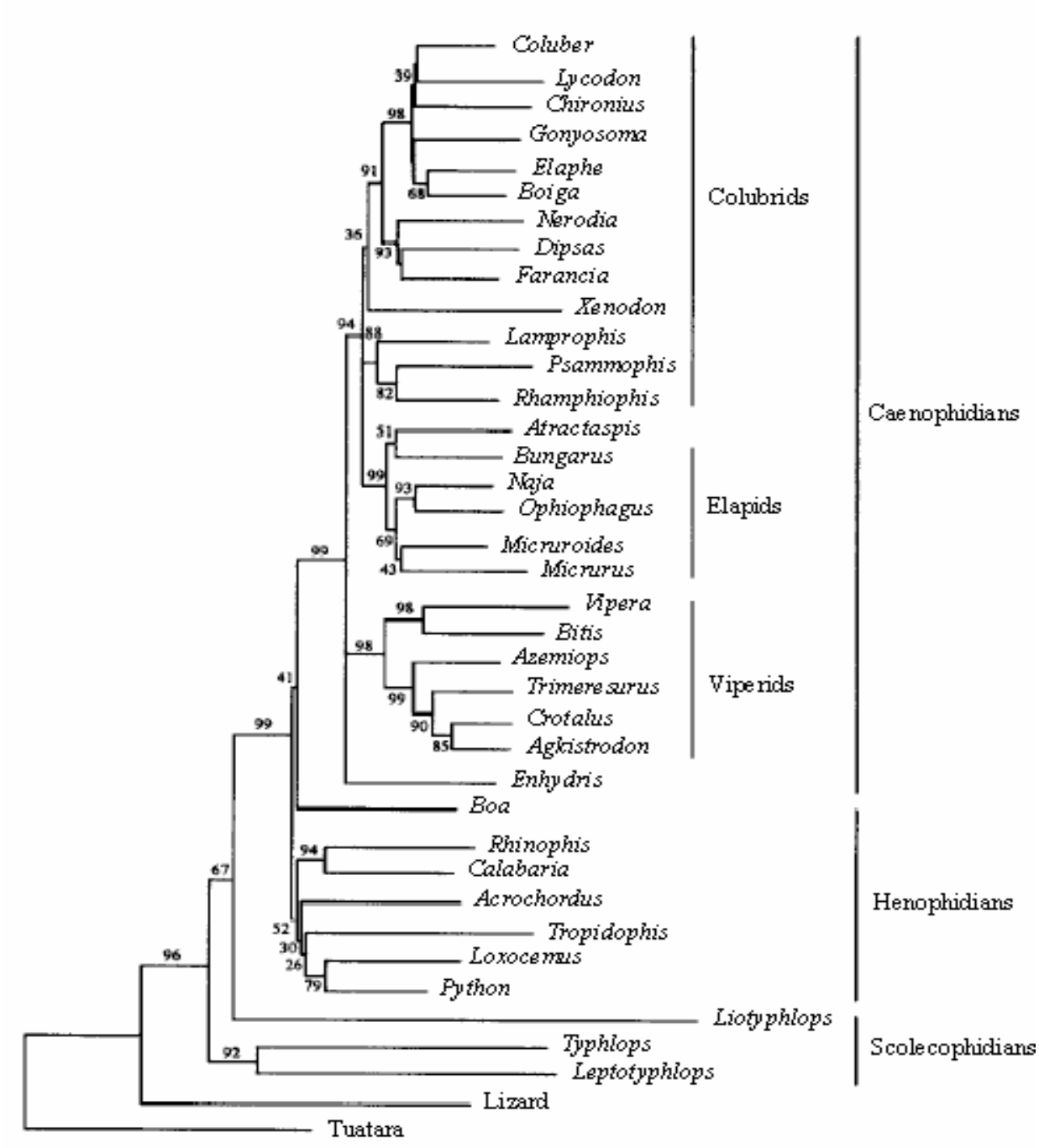
APPENDIX 1

Phylogenetic tree of snakes based on mitochondrial 12S rRNA and 16S rRNA sequence data (Heise et al 1995)[1].



[1] Heise PJ, Maxson LR, Dowling HG, Hedges SB. Higher-level snake phylogeny inferred from mitochondrial DNA sequences of 12S rRNA and 16S rRNA genes. Mol Biol Evol, 1995, 12, p. 261, by permission of Oxford University Press.