Proteases from *Vipera lebetina* Venom Affecting Coagulation and Fibrinolysis

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Key Words

Fibrino(geno)lytic enzymes · Lebetase · Factor X activator · Factor V activator · Proteases · Snake venom · *Vipera lebetina* · Glycoprotein · MALDI-TOF MS · Substrate specificity

Abstract

Our studies of the venom from the Levantine viper *Vipera lebetina* have demonstrated the existence of both coagulants and anticoagulants in the same venom. We showed that *V. lebetina* venom contains: (1) proteases that degrade fibrinogen, but not fibrin; (2) fibrinolytic enzyme (lebetase); (3) factor X activator (VLFXA); (4) factor V activator (VLFVA). Fibrinolytic enzyme and VLFXA are metalloproteases; the other studied enzymes are serine proteases. α -Fibrinogenase has no homolog among known serine proteases. β -Fibrinogenase is a typical thermostable arginine esterase that hydrolyzes esters and amides of

arginine and attacks the β -chain of fibrinogen. Lebetase is a direct-acting fibrinolytic zinc metalloendopeptidase related in amino acid sequence to repolysins. We used the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry technique for the recovery and identification of peptides released by protease hydrolysis and for the detection of human factor X cleavage products after VLFXA hydrolysis. VLFXA cleaves the Arg⁵²-Ile⁵³ bond in the heavy chain of human factor X and the Arg²²⁶-Val²²⁷ bond in human factor IX precursor; VLFVA cleaves Arg¹⁵⁴⁵-Ser¹⁵⁴⁶ in factor V.

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Introduction

Snake venoms, particularly those belonging to the Crotalidae and Viperidae families, contain a large variety of proteases that induce alterations in the blood coagulation

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0301-0147/01/0316-0123\$17.50/0

Accessible online at:
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cascade [1–8]. These highly specific proteases which cleave limited bond(s) in the blood coagulation factors are usually divided into two groups: (1) metalloproteases which need Ca^{2+} or Zn^{2+} (or both) for their hydrolytic activity and are inhibited by metal-chelating agents (factor X activator, prothrombin activator, $\alpha(\beta)$ -fibrinogenases), (2) serine proteases (factor V activator, protein C activator, plasminogen activator, kinin-releasing and thrombin-like enzymes, β -fibrinogenases). Recent studies revealed that some snake venoms contain factor X activators and prothrombin activators that are serine proteases [8].

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

V. lebetina venom contains different metalloproteases – hemorrhagic and nonhemorrhagic (including fibrinolytic enzyme, lebetase [9–15]) and factor X activator (VLFXA) [16]. Snake venom metalloproteases have homologous sequences and a typical active site structure for reprotolysins HEXXHXXGXXH [17]. The amino acid sequence of lebetase was deduced from its cDNA. The primary structure of lebetase shows extensive sequence homology with fibrolase and several other small snake venom metalloproteinases [11].

However, the substrate specificity of snake venom metalloproteases is rather different. Lebetase, a metalloprotease with thrombolytic activity, is a direct-acting fibrinolytic agent,

as it acts *via* direct cleavage of fibrin, not by plasminogen activation. In general, the fibrin-(ogen)olytic enzymes cleave off fragments from the C-terminals of α - and β -chains of fibrinogen, rendering it unclottable by thrombin. Fibrin is dissolved by the same mechanism of cleavage. Lebetase has the typical active site for reprotolysins. The enzyme readily hydrolyzes the A α chain and more slowly the B β chain of fibrinogen. Previous studies performed on the B chain of oxidized insulin showed that lebetase has primary specificity for hydrophobic P1' residues, such as Leu (Ala¹⁴-Leu¹⁵, Tyr¹⁶-Leu¹⁷) [10]. It was demonstrated that lebetase cleaves the 'bait' region in α_2 -macroglobulin and hydrolyzes pregnancy zone protein (PZP) [14]. In order to define the substrate specificity of the lebetase proteolytic activity more clearly, different peptides were used as substrates.

Furthermore, lebetase is an anticoagulant that acts by inhibiting platelet aggregation. It is a zinc-metalloproteinase with three disulfide bridges [13].

V. lebetina venom is able to activate factor X [16]. Factor X (substrate for factor X activator) is a vitamin-K-dependent protein that occupies a pivotal position in the coagulation process. Factor X is essential for hemostasis since the reduction in its functional activity results in a bleeding disorder known as Stuart-Prower factor deficiency. During physiological hemostasis, factor X can be activated by factor IXa, requiring Ca^{2+} , phospholipid, and factor VIIIa, or by factor VIIa, requiring Ca^{2+} and tissue factor. Factor X is activated to the serine protease factor Xa. The activation results from the cleavage of the Arg⁵²-Ile⁵³ bond in the heavy chain of human factor X and release of a 52-residue activation peptide [8].

The enzymes degrading the β -chain of fibrinogen without fibrinolysis belong to serine proteinases. *V. lebetina* β -fibrinogenase is a typical representative of arginine esterases

without caseinolytic activity [18]. The alkaline serine protease – a nonfibrinolytic α -fibrinogenase – has a unique specificity hydrolyzing casein but not arginine esters [19]. Factor V activator from *V. lebetina* venom (VLFVA) resembles the activator of *Vipera russelli* venom [20, 21].

Due to the potential use of snake venom proteases as diagnostic and thrombolytic agents, it is important to know their specificity against biologically active proteins and peptides. In this report we studied the specificity of *V. lebetina* proteinases. The substrates used were biologically active peptides and 6–10 amino acid residues containing peptides synthesized according to the literature provided protease cleavage regions in proteins, such as human factor X, factor IX, factor V, fibrinogen, α_2 -macroglobulin and PZP. Peptides synthesized according to the amino acid sequence of lebetase proprotein and mature protein region were also used as substrates for metalloproteases.

Sequence analysis of peptide fragments after enzyme hydrolysis has been the traditional method of determining the cleavage sites in peptides and proteins, but it is a time-consuming and rather expensive method. The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) methodology is sensitive and quick, which has led us to use this technology to obtain the primary information on the enzyme cleavage sites in peptides.

Materials and Methods

Materials

Bradykinin and substance P were purchased from Serva (Heidelberg, Germany). Ferulic acid, 2,5-dihydroxybenzoic acid (DHB) and human factor X (lot 27H93059) were from Sigma (St. Louis, Mo., USA). All other reagents used were of analytical grade.

Methods

Purification of Proteases. The purification of proteases has been described in our previous studies [10, 12, 18–20]. Enzymes were analyzed by SDS-PAGE, isoelectric focusing, HPLC and MALDI TOF MS.

Carbohydrate Test. Neutral sugars were determined by the phenol-sulfuric acid method [22], using a standard solution containing *D*-glucose. Hexosamines were assayed by the procedure of Winzler [23] after acid hydrolysis in 3 *N* HCl for 4 h at 100°C. Glucose amine was used as a standard. Neuraminic acid was measured by the thiobarbituric acid method of Warren [24] using *N*-acetylneuraminic acid as a standard.

Fibrinogen Degradation. Specific cleavage of fibrinogen was shown on 5–15% polyacrylamide gels. 0.5 ml of 1% fibrinogen solution was incubated with 25 μ g of enzyme at 37°C in 0.05 *M* Tris-saline buffer (pH 7.4). At various time intervals, 50- μ l aliquots were withdrawn and added to 50 μ l of denaturing solution (10 *M* urea, 4% SDS, 4% 2-mercaptoethanol). The samples were reduced and denatured overnight at 37°C before being electrophoresed.

Enzyme Activities. Caseinolytic activity was assayed by the method of Kunitz as modified by Mebs [25]. Azocaseinolytic activity was estimated according to the method of Charney and Tomarelli [26]. Fibrinolytic activity was estimated by the fibrin-plate method of Astrup and Müllertz [27].

Kinin-releasing activity was determined using heated human plasma (56°C, 3 h) as substrate. Plasma was centrifuged and dialysed against 0.05 *M* ammonium acetate. 5–10 μ l of enzyme solution (1 mg/ml) was added to 200 μ l of treated plasma containing 1 mM *o*-phenanthroline and the mixture was incubated at 37°C for 20 min. The mixture was ultrafiltrated (Ultra spin ultrafilters molecular weight cut-off 10,000). The kinin was detected in filtrate by MALDI-TOF MS. Arginine esterase activity was detected by the method of Schwert and Takenaka [28], using BAEE as substrate.

The activation effect of factor X by VLFXA was measured by the amidolytic activity of the factor Xa that was formed according to the method described by Hofmann and Bon [29]. Bovine or human factor X was used as substrate for VLFXA. Factor Xa activity was determined with S-2222 or S-2337 by recording the liberation of *p*-nitroaniline at 405 nm. The degradation products of human factor X treated with VLFXA were detected by MALDI TOF MS.

Molecular Mass of Enzymes

SDS-PAGE was carried out in 10 and 12.5% gels and gradient gels. The following molecular mass indi-

cators were used: bovine serum albumin (67 kD), ovalbumin (43 kD), bovine carbonic anhydrase (29 kD), soybean trypsin inhibitor (20.1 kD), cytochrome C (12.3 kD). Staining was performed with Coomassie Brilliant Blue R250.

MALDI-TOF MS

The MALDI mass spectra were measured with a home-built gridless TOF MALDI MS designed for maximum flexibility in use (National Institute of Chemical Physics and Biophysics) [13]. Cytochrome C and bovine carbonic anhydrase were used for mass calibration. Ferulic acid was used as matrix.

Peptide Synthesis. All peptides were synthesized at the 100- μ mol scale on Applied Biosystems 431A Peptide Synthesizer using BOC (t-butyl-oxycarbonyl) chemistry, as suggested by the manufacturer.

The purity of peptides was assessed by analytical reverse-phase-high-performance liquid chromatography (HPLC) and MALDI-TOF MS.

Monitoring of the Enzymatic Reaction: MALDI-TOF MS of Peptides. Peptide and peptide cleavage analyses by MALDI-TOF MS have been previously reported [15]. All peptide solutions were directly prepared in 0.1 M NH_4HCO_3 , at concentrations of about 1–5 mg/ml, and kept frozen at -20°C until use. The enzymatic hydrolysis of peptides was carried out in 0.1 M NH_4HCO_3 , at 37°C in an Eppendorf tube. In a typical experiment, 100 μl of 0.1 M NH_4HCO_3 solution of substrate (1 mg/ml) in an Eppendorf tube was thermally equilibrated to 37°C in a thermostated rack. The reaction was started by addition of 15 μl of enzyme solution (1 mg/ml in 0.1 M NH_4HCO_3). At predetermined time intervals (5 min, 0.5 h, 20 h), the aliquot (10 μl) was diluted with 100 μl of H_2O and 2 μl of 6 N HCl was added to stop the reaction. One half microliter of diluted mixture was used for MALDI-TOF MS. The matrix used for peptide analyses was 2,5-dihydroxybenzoic acid (DHB). 10 mg of DHB was dissolved in 1 ml of a 1:1 mixture of 0.1% trifluoroacetic acid and acetonitrile for sample preparation. 0.5 μl of this mixture was deposited on a stainless steel probe tip, mixed there with 0.5 μl of reaction mixture of peptides and enzyme and allowed to dry at room temperature. External mass calibration was accomplished by using as peptide standards, substance P and Lys-bradykinin.

For peptide analysis, the mass spectrometer was operated in reflectron mode with 2.6-kV pulsed extraction, 14.6 kV total acceleration voltage and 500 ns delay between laser and extraction pulse. A double multichannel plate detector was used for ion detection.

The cleavage products of human factor X after treating with VLFXA were detected by MALDI-TOF MS. The enzymatic hydrolysis of human factor X (75 $\mu\text{g}/\text{ml}$, 25 μl) was carried out in 0.1 M $\text{NH}_4\text{CH}_3\text{COO}$ containing 0.025 mM CaCl_2 (5 μl) pH 8.0, 37°C . We applied a high enzyme-substrate ratio (1:20 on a weight basis) to determine the cleavage. At predetermined time intervals (5 min, 0.5 h, 20 h), the reaction mixture (25 μl) was frozen to stop the reaction. Before MALDI-TOF analysis, samples were purified from salts using ZipTip C_4 or ZipTip C_{18} according to Millipore instructions. The matrix used for human factor X, VLFXA and cleavage products of factor X was ferulic acid, cytochrome C was used for mass calibration.

Results and Discussion

V. lebetina venom contains serine (α -fibrinogenase, β -fibrinogenase, factor V activator) and metalloproteinases (fibrinolytic enzyme lebetase, factor X activator) that affect coagulation and fibrinolysis. Molecular masses, detected by MALDI TOF MS and SDS-PAGE, are provided in table 1. Most *V. lebetina* proteases (except lebetase) are glycosylated (table 2). Two glycosylated serine fibrinogenases and VLFVA have homologous N-terminal sequences:

α -fibrinogenase	VIGGRPCNINQHRSLALLY,
β -fibrinogenase	VVGGDECNKNEHRSLVFLY,
VLFVA	VVGGDECNDINEHPFLVALY,

but can be clearly differentiated according to substrate specificity, glycosylation levels, molecular mass and fibrinogen degradation. α -Fibrinogenase has no homolog among known serine proteinases. It has N-terminal similarity with snake venom arginine esterases, but does not hydrolyze the esters of arginine, lysine and tyrosine. The enzyme has strong caseinolytic activity and degrades α -chain of fibrinogen [31] altering its clottability by thrombin. β -Fibrinogenase is a typical arginine esterase which hydrolyzes esters and amides of arginine and attacks the β -chain of fibrinogen [18]. VLFVA hydrolyzed human

Table 1. Molecular masses of *V. lebetina* venom proteases

Enzyme	Nonreduced (MALDI) D	Reduced (SDS) D
Lebetase	22,719; 22,912 [13]	23,700 [10]
VLFXA	86,500–91,000	57,500; 17,400; 14,500
VLFVA	28,400 [20]	30,000 [20]
β -Fibrinogenase	42,200 [31]	52,500 [18]
α -Fibrinogenase	31,100 [31]	37,000 [19]

Table 2. Carbohydrate composition of *V. lebetina* venom proteases

Enzyme	Hexoses, %	Hexosamines, %	Sialic acid, %
VLFXA	8.0	2.5	2.5
VLFVA	4.4	4.4	3.1
α -Fibrinogenase	8.5	5.8	0.8
β -Fibrinogenase	23.0	8.3	1.0

factor V peptide fragments, containing 7–9 amino acids (YLRSNNG, WYLRSNNG and AWYLRSNNG), synthesized according to *V. russelli* factor V activator cleavage region in human factor V. VLFVA cleaves Arg-Ser bond in these peptides (fig. 1). We may suppose that VLFVA cleaves Arg¹⁵⁴⁵-Ser¹⁵⁴⁶ bond in human factor V. We have shown earlier that *V. lebetina* venom contains two different bradykinin-releasing serine enzymes [30]. The released bradykinin from plasma was detected in this work by MALDI TOF MS (fig. 2). This method successfully replaces the rat uterus test formerly used for kinin detection.

Specificity of metalloproteinases was studied against different proteins and peptides. Lebetase specificity was characterized in our previous publications [10, 13–15]. Lebetase hydrolyzed peptides synthesized according to the proprotein and mature protein region of Le-3 (table 3). Le-3 designates the deduced amino acid sequence of the precursor for lebetase [11]. Lebetase is synthesized in the veno-

mous gland in a latent zymogen form containing prosequences. The mature protease may be released autocatalytically. The Le-3 fragment is also hydrolyzed by some other *V. lebetina* proteases from I–II fraction of Sephadex G-100 gel filtration.

Comparison of amino acid sequences for a variety of substrates shows that lebetase has no strict P1-P1' specificity requirement (table 3). Hydrolysis at sites with a Pro residue at P1 was observed with bradykinin, substance P, peptide fragments of PZP and fibrinogen α -chain as substrates.

The purified VLFXA had no effect on fibrinogen, prothrombin, plasminogen, indicating that it might activate factor X specifically. The effect of VLFXA on human factor X was studied by measuring the amidolytic and the coagulant activities of the activated factor X (factor Xa). The factor-X-activating enzyme VLFXA has no amidolytic activity against factor Xa substrates S-2337 or S-2222, while *V. lebetina* venom itself hydrolyzes

Fig. 1. a MALDI TOF mass spectrum of human factor V peptide fragment 1543–1549 YLRSNNG (823.4 D). **b** Mass spectrum of cleavage products (YLR 451.3 D, SNNG 391.2 D) of human factor V peptide fragment 1543–1549 after treatment with VLFVA.

these substrates. The activator converts the inactive factor X to the active form Xa in the presence of Ca^{2+} ions. The activation without Ca^{2+} was about 5%. The enzyme has maximum activity on factor X in the interval of pH 7.5–8.0.

VLFXA hydrolyzed 6–9 amino acid residues containing peptide fragments (TRIVGG, LTRIVGG and NNLTRIVGG), synthesized according to the physiological cleavage region of human factor X. VLFXA cleaved Arg-Ile bond in peptides. NNLTRI-

Fig. 2. MALDI TOF mass spectrum of bradykinin (1,060.6 D) liberated after 0.5 h of treatment of heated human plasma at 37°C with bradykinin-releasing enzyme fraction. A contaminating component with a mass of 1,076.6 D was found in nontreated plasma. Details are provided in Methods.

Table 3. Hydrolysis of peptide substrates by lebetase

Name of substrate	Cleavage P1↓P1'
Substance P	RP↓KP↓QQFFG↓LM* [15]
Bradykinin	RPPGFSP↓FR [15]
Le-3 fragment 192–198	TPE↓Q↓QRF
Le-3 fragment 191–198	LTPE↓Q↓QRF
PZP fragment 686–693	PYVP↓QLGT [15]
α ₂ -M fragment 676–684	GPEG↓LRVGF [15]
α ₂ -M fragment 693–700	GHAR↓LVHV [15]
Fibrinogen fragment 513–520	FFSP↓MLGE [15]
Fibrinogen fragment 408–417	EYHTEK↓LVTS [15]

↓ = Cleavage site of peptide by lebetase; *P↓Q is the main cleavage site (after 5 min hydrolysis) of substance P by lebetase.

VGG was cleaved most effectively. Activator hydrolyzed 9 amino acid residues containing peptide fragment-NDFTRVVGG, synthesized according to the physiological cleavage region of human factor IX, in position Arg-Val (fig. 3). These peptides were used for

localization of VLFXA in different steps of purification and also in inhibitory tests.

The catalytic cleavage of human factor X by the activators from *V. lebetina* (VLFXA) and *V. russelli* (RVVXAE, Sigma) venoms was examined by MALDI-TOF MS. The

Fig. 3. a MALDI TOF mass spectrum of human factor IX peptide fragment 222–230 NDFTRVVG (964.5 D), contaminating component – N-terminus cyclization product of peptide. **b** Mass spectrum of cleavage products (NDFTR 651.7 D, VVG 331.2 D) of human factor IX peptide fragment after treating with VFXA.

masses of cleavage products of human factor X were 43.1 and 11.6 kD after treating with VFXA and RVV-X. The activation was already detectable after 5 min of hydrolysis in the case of VFXA. On the basis of cleavage of peptides and human factor X, we can con-

clude that VFXA hydrolyses human factor X at the positions Arg⁵²-Ile⁵³ and human factor IX precursor at the positions Arg²²⁶-Val²²⁷. Factor X can be completely converted to factor Xa by VFXA and the specific activity of factor Xa is the same as that of factor Xa

obtained after activation by the activator from Russell's viper venom [32].

The use of MALDI-TOF MS has several advantages over traditional methods for the elucidation of cleavage sites by proteases in peptides and proteins. MALDI-TOF MS has high sensitivity that allows the analysis of small aliquots removed from reaction mixture. Our results show that MALDI-TOF MS is a very informative tool and can easily characterize the composition of cleaved peptides

and proteins. However, as it stands now, MALDI is not quantitative enough for detecting kinetic parameters of enzyme reactions.

Acknowledgements

We are grateful to Dr. Nisse Kalkkinen (Institute of Biotechnology, University of Helsinki) for performing the N-terminal analysis. The work was financially supported by Estonian Science Foundation Grants No. 4228 and No. 3842.

References

- Kornalik F: The influence of snake venom enzymes on blood coagulation. *Pharmacol Ther* 1985;29:353–405.
- Kornalik F: Toxins affecting blood coagulation and fibrinolysis; in Shier WT, Mebs D (eds): *Handbook of Toxinology*. New York, Marcel Dekker, 1990, pp 683–759.
- Seegers WH, Ouyang C: Snake venoms and blood coagulation; in Lee CY (ed): *Snake Venoms, Handbook of Experimental Pharmacology*. Berlin, Springer, 1979, vol 52, pp 684–750.
- Stocker K: Snake venom proteins affecting hemostasis and fibrinolysis; in Stocker K (ed): *Medical Use of Snake Venom Proteins*. Boca Raton, CRC Press, 1990, pp 97–160.
- Ouyang C, Teng CMT, Huang F: Characterization of snake venom components acting on blood coagulation and platelet function. *Toxicon* 1992;30:945–966.
- Braud S, Bon C, Wisner A: Snake venom proteins acting on hemostasis. *Biochimie* 2000;82:851–859.
- Matsui T, Fujimura Y, Titani K: Snake venom proteases affecting hemostasis and thrombosis. *Biochim Biophys Acta* 2000;1477:146–156.
- Morita T: Proteases which activate factor X; in Bailey GS (ed): *Enzymes from Snake Venom*. Alaken, Fort Collins, 1998, pp 179–208.
- Siigur E, Siigur J: The direct-acting α -fibrin(ogen)olytic enzymes from snake venoms. *J Toxicol Toxin Rev* 1992;11:91–113.
- Siigur E, Siigur J: Purification and characterization of lebetase, a fibrinolytic enzyme from *Vipera lebetina* (snake) venom. *Biochim Biophys Acta* 1991;1074:223–229.
- Siigur E, Aaspõllu A, Tu AT, Siigur J: cDNA cloning and deduced amino acid sequence of fibrinolytic enzyme (lebetase) from *Vipera lebetina* snake venom. *Biochem Biophys Res Commun* 1996;224:229–236.
- Siigur J, Tõnismägi K, Tu AT, Siigur E: Cross-reactivities of polyclonal antibodies against lebetase, fibrinolytic enzyme of Levantine viper (*Vipera lebetina*) venom. *Toxicon* 1996;34:608–613.
- Siigur J, Samel M, Tõnismägi K, Subbi J, Siigur E, Tu AT: Biochemical characterization of lebetase, a direct-acting fibrinolytic enzyme from *Vipera lebetina* snake venom. *Thromb Res* 1998;90:39–49.
- Saidi N, Samel M, Siigur J, Jensen PEH: Lebetase, an $\alpha(\beta)$ -fibrin(ogen)olytic metalloproteinase of *Vipera lebetina* snake venom, is inhibited by human α -macroglobulins. *Biochim Biophys Acta* 1999;1434:94–102.
- Trummel K, Vija H, Subbi J, Siigur J: MALDI-TOF mass spectrometry analysis of substrate specificity of lebetase, a direct-acting fibrinolytic metalloproteinase from *Vipera lebetina* snake venom. *Biochim Biophys Acta* 2000;1476:331–336.
- Siigur J, Samel M, Tõnismägi K, Siigur E: Factor X activators from *Viperidae* venoms. *Toxicon* 1995;33:258.
- Bjarnason JB, Fox JW: Snake venom metalloendopeptidases: Reprolysins. *Methods Enzymol* 1995;248:345–368.
- Siigur E, Mähar A, Siigur J: β -Fibrinogenase from the venom of *Vipera lebetina*. *Toxicon* 1991;29:107–118.
- Mähar A, Siigur E, Siigur J: Purification and properties of a proteinase from *Vipera lebetina* (snake) venom. *Biochim Biophys Acta* 1987;925:272–281.
- Siigur E, Samel M, Tõnismägi K, Subbi J, Reintamm T, Siigur J: Isolation, properties and N-terminal amino acid sequence of a factor V activator from *Vipera lebetina* (Levantine viper) snake venom. *Biochim Biophys Acta* 1998;1429:239–248.
- Siigur E, Aaspõllu A, Siigur J: 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* 1999;262:328–332.
- Dubois M, Gilles KA, Hamilton KK, Rebers PA, Smith F: Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956;28:350–356.
- Winzler RJ: Determination of serum glycoproteins; in Glick D (ed): *Methods of Biochemical Analysis*. New York, Interscience, 1958, vol 2, pp 279–311.

- 24 Warren OL: The thiobarbituric acid assay of sialic acids. *J Biol Chem* 1959;234:1971–1975.
- 25 Mebs D: A comparative study of enzyme activities in snake venoms, *Int J Biochem* 1970;1:335–342.
- 26 Charney J, Tomarelli RM: A colorimetric method for the determination of the proteolytic activity of duodenal juice. *J Biol Chem* 1947;131: 501–505.
- 27 Astrup T, Müllertz S: The fibrin plate method for estimating of fibrinolytic activity. *Arch Biochem Biophys* 1952;40:346–351.
- 28 Schwert GW, Takenaka Y: A spectrophotometric determination of trypsin and chymotrypsin. *Biochim Biophys Acta* 1955;16:570–575.
- 29 Hofmann H, Bon C: Blood coagulation induced by the venom of *Bothrops atrox*. 2. Identification, purification and properties of two factor X activators. *Biochemistry* 1987;26: 780–787.
- 30 Siigur EP, Siigur JR, Aaviksaar AA, Kibirev VK, Fedoryak DM: Separation of a bradykinin-releasing enzyme from the proteolytic complex of Levantine viper (in Russian). *Biokhimiya* 1982;47:1730–1737.
- 31 Samel M, Subbi J, Siigur J, Siigur E: Biochemical characterization of fibrinogenolytic serine proteinases from *Vipera lebetina* snake venom. *Toxicon* 2002;40:51–54.
- 32 Takeya H, Nishida S, Miyata T, Kawada S, Saisaka Y, Morita T, Iwanaga S: Coagulation factor X activating enzyme from Russell's viper venom (RVV-X). A novel metalloproteinase with disintegrin (platelet aggregation inhibitor)-like and C-type lectin-like domains. *J Biol Chem* 1992;267:14109–14117.