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Research paper

A new tyrosine-specific chymotrypsin-like and angiotensin-degrading serine proteinase from *Vipera lebetina* snake venom☆

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ABSTRACT

Vipera lebetina venom contains different metallo- and serine proteinases that affect coagulation and fibrin(ogen)olysis. A novel serine proteinase from ***V. L****ebetina* venom having **C**hymo**T**rypsin **L**ike **P**roteolytic activity (**VLCTLP**) was purified to homogeneity from the venom using Sephadex G-100sf, DEAE-cellulose, heparin–agarose and FPLC on Superdex 75 chromatographies. VLCTLP is a glycosylated serine proteinase with a molecular mass of 41926 Da. It reacts with N-acetyl-L-tyrosine ethyl ester (ATEE) but not with Suc-Ala-Ala-Pro-Phe-pNA or Suc-Ala-Ala-Pro-Leu-pNA. The complete amino acid sequence of the VLCTLP is deduced from the nucleotide sequence of the cDNA encoding this protein. The full-length cDNA sequence of the VLCTLP encodes open reading frame of 257 amino acid residues that includes a putative signal peptide of 18 amino acids, a proposed activation peptide of six amino acid residues and serine proteinase of 233 amino acid residues. VLCTLP belongs to the S1 (chymotrypsin) subfamily of proteases. The multiple alignment of its deduced amino acid sequence showed structural similarity with other serine proteases from snake venoms. The protease weakly hydrolyses azocasein, Aα-chain and more slowly Bβ-chain of fibrinogen. VLCTLP does not cleave fibrin and has no gelatinolytic activity. Specificity studies against peptide substrates (angiotensin I and II, oxidized insulin B-chain, glucagon, fibrinogen fragments etc.) showed that VLCTLP catalysed the cleavage of peptide bonds after tyrosine residues. VLCTLP is the only purified and characterized serine proteinase from snake venoms that catalyses ATEE hydrolysis. We detected ATEE-hydrolysing activities also in 9 different Viperidae and Crotalidae venoms.

1. Introduction

Snake venoms are rich sources of biologically active components including enzymes and nonenzymatic proteins and peptides, which can be used in studying basic mechanisms of haemostasis and envenomation to enable us to develop diagnostic and therapeutic agents. Snake venoms, particularly those belonging to the Crotalidae and Viperidae families, are a rich source of proteolytic enzymes that have been extensively investigated [reviews 1–6]. Proteomic (venomics) analyses have also proved the presence of various proteinases in the venoms of Viperidae and Crotalidae snakes [7–9].

These highly specific proteinases which cleave limited bond(s) in the blood coagulation factors are usually divided into two groups: 1) metalloproteinases–reprolysins which need Zn^{2+} or Ca^{2+} (or both) for their lytic activity and are inhibited by metal chelating agents (fibrinolytic enzymes, hemorrhagic proteinases, factor X activator) [review 10]; 2) serine proteinases [e.g. thrombin-like enzymes that catalyse the cleavage of fibrinogen, liberating fibrinopeptides A and/or B (A, B and AB venombins), factor V activator, protein C activator, plasminogen activator, kinin-releasing enzymes, α- and β-fibrinogenases]. The snake venom serine proteinases contain a highly conserved catalytic triad comprising of residues His57, Asp102 and Ser195 (chymotrypsinogen numbering) [reviews 1,2,5,11]. We have earlier shown that *Vipera lebetina* venom contains all four classes of snake venom metalloproteases [12] and different serine proteases [13–15] that affect coagulation and fibrin(ogen)olysis. Snake venom serine proteases usually have trypsin-like and thrombin-like activities hydrolysing p-toluenesulfonyl-L-arginine methyl ester (TAME) and N-benzoyl-L-arginine ethyl ester (BAEE). Tu et al. [16] tested chymotrypsin-like activity of thirty-three Crotalidae, Viperidae and Elapidae venoms using N-benzoyl-L-tyrosine ethyl ester (BTEE) and N-acetyl-L-tyrosine ethyl ester (ATEE) but failed to detect tyrosine

☆ The sequences data of VLCTLP and VLBF reported in this paper have been submitted to the GenBank under accession nos. GU570565 and GU570566, respectively.

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esterase activity in the majority of these venoms except those of *Agkistrodon halys* and *Bothrops jararaca* [16].

In this paper we describe the purification and characterize a novel stable monomeric serine proteinase in ***V. L****ebetina* venom having tyrosine-specific **C**hymo**T**rypsin **L**ike **P**roteolytic activity (**VLCTLP**). We also present the complete amino acid sequence of VLCTLP deduced from cDNA. We detected ATEE-hydrolysing activities in 9 different Viperidae and Crotalidae venoms.

2. Materials and methods

2.1. Materials

2,5-Dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid, oxidized insulin B-chain from bovine pancreas, glucagon from hog pancreas, angiotensin I and II, azocasein, bovine fibrinogen, thrombin, gelatin, Substance P, Cytochrome C, N-Suc-Ala-Ala-Pro-Leu-pNA and N-Suc-Ala-Ala-Pro-Phe-pNA were from Sigma–Aldrich (USA), trypsin from Promega, USA, PNGase F from New England Biolabs (USA). Sephadex G-100 (superfine) was the product of Pharmacia (Uppsala, Sweden), Superdex 75 – Amersham Biosciences (UK), DEAE-cellulose DE-52 (Whatman, UK). Heparin–agarose was obtained from Kemotex Bio (Tallinn, Estonia). BAEE and ATEE were from Reanal (Hungary). Oligonucleotide primers were ordered from DNA Technology (Aarhus, Denmark). The studied venoms originate from Tashkent Integrated Zoo Plant, Uzbekistan (*V. lebetina*, *Vipera ursini*, *Naja oxiana*), from Latoxan, France (*Vipera russellii russellii, Echis ocellatus*, *Cerastes cerastes*, *Bitis arietans*, *Agkistrodon bilineatus*, *Calloselasma rhodostoma*, *B. jararaca*, *B. lanceolatus, Oxyuranus scutellatus*), from Astik Farm, India (*Bungarus caeruleus, E. carinatus, N. naja*), from Tripolski Zooplant, Ukraine (*A. halys, Agkistrodon blomhoffi, Agkistrodon saxatilis, Naja atra*), from Sigma, USA (*Agkistrodon contortrix contortrix*), from Khimky Serpentarium, Russia (*Vipera berus berus*).

All other reagents used were of analytical grade.

2.2. Purification of the enzyme

Crude venom (1.5 g) was dissolved in 10 ml of 0.2 M ammonium acetate, pH 6.7. Insoluble material was removed by centrifugation (5000×*g* for 15 min) and the supernatant was applied to the column (2.2 × 140 cm) of Sephadex G-100 superfine equilibrated with 0.2 M ammonium acetate. The elution was carried out with the same solution at a flow rate of 4 ml/h and 6 ml fractions were collected at 4 °C. The absorbance was continuously monitored at 280 nm. Fractions I-VIII were pooled as shown in Fig. 1A and lyophilized.

Combined and concentrated by lyophilization, fraction III from gel filtration (50 mg in 2 ml of 0.1 M ammonium bicarbonate) was applied onto the Whatman DE-52 cellulose column (1.5 × 21 cm) equilibrated with 0.1 M ammonium bicarbonate. Non-adsorbed material was washed with the equilibration solution. The column was eluted with the linear gradient of 0.1–0.5 M ammonium bicarbonate, flow rate 12 ml/h; fractions of 4.8 ml were collected.

7.7 mg of lyophilized material (fractions with chymotrypsin-like activity from DEAE-cellulose) was dissolved in 1 ml of 0.05 M ammonium bicarbonate and applied to the column of heparin–agarose (1.5 × 14 cm) equilibrated with 0.05 M ammonium bicarbonate. The column was eluted with 0.1 M ammonium bicarbonate and 2 M ammonium bicarbonate. The flow rate was 6 ml/h; fractions of 3 ml were collected.

The lyophilized VLCTLP fraction with ATEE-hydrolysing activity from heparin–agarose column (0.5 mg) was dissolved in 0.1 ml of

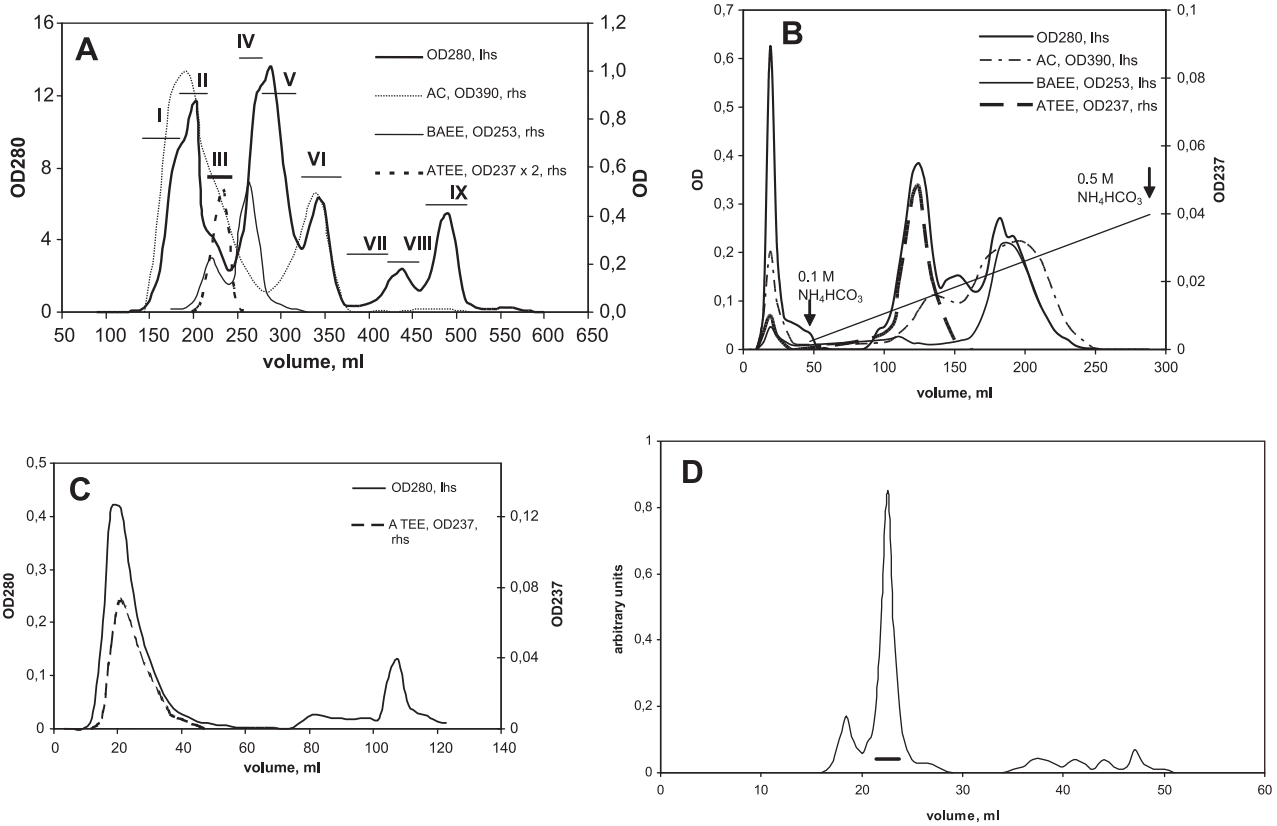

Fig. 1. Purification of VLCTLP from the *Vipera lebetina* venom (A) Gel filtration of *V. lebetina* venom. (B) Ion exchange chromatography on DE-52 cellulose. (C) Heparin–agarose chromatography. (D) Gel filtration on FPLC Superdex 75 column. The measured ATEE activity is shown in bold line (lhs – left hand side; rhs – right hand side; AC – azocasein).

0.2 M ammonium acetate and applied to the Superdex 75 (10/300) column equilibrated with 0.2 M ammonium acetate. The elution was carried out with the same solution. Protein concentrations were determined using the Pierce micro BCA kit, and bovine serum albumin was used as a standard. During the process of column chromatography the elution profile was followed by the absorbance at 280 nm.

2.3. Protease and esterase activities

Esterolytic activity with BAEE (0.25 mM; 0.05 M Tris–HCl, pH 8.5) and ATEE (0.5 mM; 0.05 M Tris–HCl, pH 8.5) was determined spectrophotometrically according to Schwert and Takenaka [17]. To 1 ml of substrate 20 μl of the enzyme (1 mg/ml) was added and the increase in absorbance at 253 nm in case of BAEE and the decrease in absorbance at 237 nm in case of ATEE was recorded. One unit is defined as the 1.0 change in absorbance in 0.05 M Tris–HCl, pH 8.5 at 25 °C per min. The activity with chymotrypsin substrates *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA) and *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (Suc-AAPL-pNA) was determined spectrophotometrically. To 1 ml of substrate (0.4 mM in 0.05 M Tris–HCl, pH 8.3) 20 μl of the enzyme (1 mg/ml) was added and the change in absorbance at 410 nm was recorded.

Azocaseinolytic activity was measured as described by Siigur et al. [18].

VLCTLP-dependent digestion of fibrinogen was assessed with bovine fibrinogen as substrate. 0.1 ml of 2% fibrinogen solution was incubated with 0.1 ml of VLCTLP (10 μg) at 37 °C in 0.05 M Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl. At various time intervals 0.025 ml of the incubation mixture was withdrawn and added to 0.025 ml of denaturing solution (10 M urea, 4% SDS, 4% 2-mercaptoethanol). The samples were incubated overnight at 37 °C before electrophoresis. Specific cleavage of fibrinogen was shown on 12.5% SDS-polyacrylamide gels. Fibrinolytic activity was determined by fibrin plate method of Astrup and Müllertz [19].

Gelatinolytic activity was determined according to Bee et al. [20]. Gelatin was co-polymerised into 10% PAA-gel at final concentration of 2.5 mg/ml. VLCTLP (5 μg) was mixed with non-reducing sample buffer and applied to the gel. After electrophoresis the SDS was removed by washing the gel for 1 h in 2.5% Triton X-100 before incubation in 50 mM Tris–buffer (50 mM $CaCl_2$, 0.2 M NaCl, 0.07% Brij 35), pH 7.6 at 37 °C for 16 h and the gel was stained with 0.125% Coomassie brilliant blue R250. Clear areas in the gel indicate regions of enzyme activity.

The thermostability was studied incubating the enzyme solution (20 μl; 1 mg/ml in 50 mM CH_3COONH_4, pH 6.5) at various temperatures (25, 37, 50, 60, 70, 80, 100 °C) for 10 min; then quickly cooled and ATEE-hydrolysing activities were determined.

2.4. Molecular mass determination and isoelectric focusing

The molecular masses of the purified proteins were determined by SDS-PAGE on 12.5% polyacrylamide gels using the method of Laemmli [21]. The molecular masses were also determined using a home-built (National Institute of Chemical Physics and Biophysics) matrix-assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF-MS) designed for maximum flexibility in use. For MALDI-TOF analysis the dried samples were dissolved in 5 μl of 50% ACN, 0.1% TFA. Aliquots of 0.5 μl were applied onto the target, allowed to air dry and 0.5 μl of the matrix solution (2,5-dihydroxybenzoic acid – DHB) was applied to the target and allowed to dry in air. The mass calibration standards were cytochrome C and insulin B-chain. A nitrogen 337 nm laser (4 ns pulse) was used and at least 30–40 shots were summed up.

Analytical isoelectric focusing was performed on 5% polyacrylamide gel plates according to the method of Vesterberg [22] in Multiphor 2117 (LKB, Sweden) apparatus in the pH range of 3.6–9.3. The gels were stained for proteins with Coomassie Brilliant Blue R250.

2.5. N-terminal sequencing

Five μg of lyophilized VLCTLP was dissolved in 10 μl H_2O and run by reversed phase chromatography on a 1 × 150 mm Jupiter C4 (5 μm, 300 Å, Phenomenex Inc, USA) using a linear gradient of acetonitrile (0–60% in 60 min) in 0.1% trifluoroacetic acid. Flow rate was 50 μl/min and detection at 214 nm. The protein peak was collected and used for sequencing by Edman degradation on a Procise 494A Sequencer (Perkin Elmer, Applied Biosystems Division, CA, USA).

2.6. Tryptic digestion and mass fingerprinting

Trypsinolysis was performed in gel as in solution.

After visualization with Coomassie Blue the gel-electrophoresis bands of protein in interest (native or reduced) were excised from SDS-polyacrylamide gels, each gel slice was cut into small pieces (1 mm^2), placed into eppendorf tube and treated as described earlier [23]. Equal volumes (0.5 μl) of the peptide mixture and the matrix (2,5-dihydroxybenzoic acid, or α-cyano-4-hydroxycinnamic acid) were mixed on the MALDI-TOF plate. The mass calibration standard was substance P.

In case of in solution trypsinolysis 5 μg of purified VLCTLP was dissolved in 50 μl of 6 M Guanidine–HCl, 0.5 M Tris–HCl, 2 mM EDTA, pH 7.5 and 5 μl of 100 mM DTT was added. After keeping the mixture for 30 min at room temperature, 6 μl of 200 mM iodoacetamide was added and the mixture was kept at room temperature for 30 min, then 5 μl of 100 mM DTT was added. The alkylated protein was desalted by reversed phase chromatography on a 1 × 20 mm TSK gel TMS-250 column (C1, 10 μm, 250 Å, 1 × 20 mm, TOSOH Corporation, Japan) as described for N-terminal sequencing. The protein peak was collected and dried by vacuum centrifugation. For trypsin digestion, the dried sample was dissolved in 50 μl of 0.1 M ammonium bicarbonate, 0.05 μg trypsin (Sequencing Grade Modified, V5111, Promega) was added and incubated at 37 °C overnight. For peptide *de novo* sequencing, 10 μl of the tryptic digest was subjected to LC-MS/MS analysis on a Q-TOF (Micromass, UK) as described before [24]. The amino acid sequences of the N-terminal and tryptic peptides were compared with the BLASTP database [25].

2.7. Deglycosylation of VLCTLP

For deglycosylation of VLCTLP PNGase F deglycosylation kit (New England Biolabs) was used. 18 μg (9 μl) of glycoprotein (VLCTLP), 1 μl of the 10× glycoprotein denaturing buffer (5% SDS, 0.4 M DTT) and 2 μl of H_2O were combined and the glycoprotein was denatured by heating at 100 °C for 10 min. The total reaction volume was made of 20 μl by adding 2 μl of 10× G7 reaction buffer (0.5 M sodium phosphate pH 7.5), 2 μl of 10% n-octylglucoside and 2 μl of PNGase F, the reaction mixture was incubated at 37 °C overnight (in case of native deglycosylation 4 μl of PNGase F and 24 h incubation) and subjected to SDS electrophoresis or trypsinolysis. In case of the latter the mixture was once again denatured with 2 μl of denaturing buffer and heated at 100 °C for 10 min. Then 30 μl of 2-iodoacetamide (10 mg/ml, Fluka) was added and the reaction mixture was held in dark for 1 h. Fifty μl of water was added, followed by 20 μl of trypsin solution (Promega Trypsin Gold, 25 ng/μl). The reaction mixture was incubated at 37 °C for 24 h. The

Table 1
Purification of VLCTLP from *Vipera lebetina* venom.

Purification step	Total protein (mg)	Specific activity (unit/mg)[a]	Total activity (units)	Purification (fold)	Recovery (% initial activity)
Crude venom	1500.0	0.2	300.0	1.0	100.0
Fraction III from gel filtration	113.3	2.6	291.3	12.9	97.1
DE-52 cellulose	18.1	6.7	120.4	33.3	40.1
Heparin–agarose	10.1	7.4	74.2	36.8	24.8
Superdex 75	6.3	9.0	56.7	45.0	18.9

[a] The activity of the enzyme was measured with N-acetyl-L-tyrosine ethyl ester (ATEE) as substrate.

sample of tryptic peptides was purified using ZipTip C_{18} and analyzed with MALDI-TOF MS.

2.8. Synthesis and hydrolysis of oligopeptide substrates

The peptide fragments 408–417 from fibrinogen A chain and 687–693 from pregnancy zone protein, PZP, were synthesized at the 100 μmol scale on Applied Biosystems 431A Peptide Synthesizer using BOC (t-butyl-oxycarbonyl) chemistry as suggested by the manufacturer. The purity of peptides was assessed by analytical reverse phase – high performance liquid chromatography (RP-HPLC) and MALDI-TOF mass spectrometry. The peptide solutions were directly prepared in 0.05 M NH_4HCO_3, at concentrations of about 1–5 mg/ml, and kept frozen at −20 °C until use.

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

2.9. Platelet aggregation assay

Platelet aggregation assays were performed in human platelet-rich plasma according to the previously described protocol [18].

2.10. PCR amplification, cloning and sequencing

The cDNA encoding VLCTLP was isolated by PCR amplification as a byproduct of cloning of the alpha-fibrinogenase-encoding cDNA. The *V. lebetina* venom gland cDNA library constructed in the Uni-ZAP XR vector [26] was used as a source of the template. Using the antisense primer 5′-GGTGTAGCTTTTGTTACTGAG-3′ (PI) and T3 as sense primer, the cDNA fragment corresponding to the 5′-terminal sequence of VLCTLP was amplified. On the basis of this sequence the primer PII (5′-CGAAATCAGGATGAGC-3′; sense) was designed for amplification of the main VLCTLP-coding cDNA fragment with T7 as antisense primer. The PCR conditions were as follows: denaturation at 94 °C – 1 min; annealing at 55 °C – 1 min: extension at 72 °C – 1 min 30 s; 35 cycles. After standard procedures some random clones were sequenced along both strains on Perkin Elmer ABI Prism Model 310 DNA Sequencer by cycle sequencing using DYEnamic™ ET Terminator Cycle Sequencing Kit (Amersham Biosciences). Three individual VLCTLP clones generated by independent PCRs were analysed. The full-length cDNA was constructed from the overlapping fragments.

3. Results

3.1. Purification and characterization

VLCTLP was purified from the venom using Sephadex G-100sf, DEAE-cellulose, heparin–agarose and FPLC Superdex 75 chromatographies.

The first step – size exclusion chromatography on Sephadex G-100sf, – enables to get rid of various enzymes such as phosphodiesterase, 5′-nucleotidase, phosphomonoesterase, L-amino acid oxidase [27], apoptosis-inducing protease [28] and factor X activator [23] (peaks I and II, Fig. 1A). The chymotrypsin-like activity (substrate ATEE) was detected in the third peak with several other components. The fourth and fifth peaks contain alpha-fibrinogenase [15], factor V activator [13], phospholipase A_2 [29], nerve growth factor [30], viplebedin-2 [31], and different other components, peak VI contains lebetase [32]. By ion exchange chromatography on DEAE-cellulose the chymotrypsin-like activity was detected in the second peak (Fig. 1B). VLCTLP could be further purified by heparin–agarose chromatography where chymotrypsin-like activity was observed in the first peak (Fig. 1C). The final purification was obtained using gel filtration on FPLC Superdex 75 column (Fig. 1D). The final preparation showed 45-fold purification of the enzyme with an 18.9% yield of the activity and 0.42% of the protein (Table 1). The content of the enzyme in venom batches is variable, 0.25–0.55% depending on the batch used. The molecular mass of VLCTLP is 55 kDa by SDS-PAGE (Fig. 2), 41926 Da by MALDI-TOF MS (Fig. 3) and after deglycosylation about 27 kDa by SDS-PAGE (Fig. 4). Deglycosylation of the native enzyme resulted in heterogeneous preparation due to incomplete process (data not shown). Isoelectric point of VLCTLP is in the acid region (≤3.6). The enzyme was stable at 4 °C for several months. The partial decrease in activity was observed after heating for 10 min at 70 °C and about 40% of activity was retained at 100 °C. Partial deglycosylation had no effect on enzyme activity (substrate ATEE).

We tested ATEE-hydrolysing activity in 22 Viperidae, Crotalidae and Elapidae venoms and the activity was observed in 9 snake

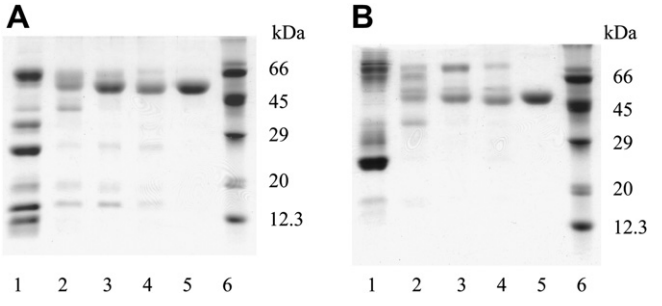

Fig. 2. SDS-PAGE (12.5% gel) of VLCTLP from different purification steps in reduced (A) and non-reduced (B) conditions: 1 – *V. lebetina* venom; 2 – fraction III from gel filtration; 3 – VLCTLP fraction from DEAE-cellulose; 4 – VLCTLP fraction from heparin–agarose; 5 – VLCTLP fraction from Superdex 75 column; 6 – molecular mass markers (bovine serum albumin 66 kDa, ovalbumin 45 kDa, carboanhydrase 29 kDa, soybean trypsin inhibitor 20 kDa, cytochrome C 12.3 kDa).

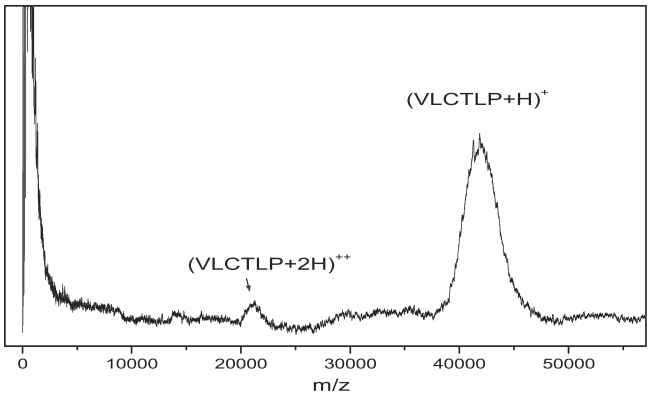

Fig. 3. MALDI-TOF mass spectrum of VLCTLP. Molecular mass 41926 Da. Matrix was ferulic acid.

venoms (see Table 2). The highest activities were in *E. ocellatus* and *V. lebetina* venoms. We could not find any chymotrypsin-like activity in the venoms of the following snakes: *A. bilineatus, A. contortrix contortrix*, *B. arietans*, *B. lanceolatus*, *C. rhodostoma*, *C. cerastes*, *E. ocellatus*, *V. russellii russellii*, *V. ursini*, *B. caeruleus, O. scutellatus, N. atra, N. oxiana*.

3.2. Substrate specificity

Substrate specificity of the VLCTLP was studied against different proteins. The enzyme hydrolyses azocasein very weakly and has no gelatinolytic activity. PMSF (1 mM, preincubation for 1 h) inhibited the hydrolytic activity of the enzyme. These experiments confirm the serine proteinase nature of the enzyme.

Incubation of VLCTLP with 1% fibrinogen prolonged the fibrinogen coagulation time by thrombin (Fig. 5B). Cleavage of fibrinogen is shown in Fig. 5A. The enzyme (50 μg/ml) digested Aα-chain, the Bβ-chain was cleaved more slowly and the γ-chain was left intact even after 24 h incubation. The fibrinogenolytic activity is remarkably weaker than that of the other fibrinogenases in the venom. The enzyme does not show fibrinolytic activity since it does

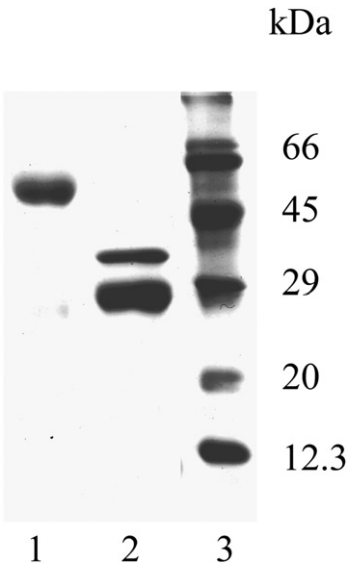

Fig. 4. Deglycosylation of VLCTLP with PNGase F. 12.5% SDS-PAGE in reduced conditions: 1 – VLCTLP; 2 – deglycosylated VLCTLP and PNGase F (36 kDa); 3 – molecular mass markers (bovine serum albumin 66 kDa, ovalbumin 45 kDa, carboanhydrase 29 kDa, soybean trypsin inhibitor 20 kDa, cytochrome C 12.3 kDa).

Table 2
ATEE esterase activities of snake venoms[a].

Species	unit/mg
Echis ocellatus	0.284
Vipera lebetina	0.200
Echis multisquamatus	0.120
Agkistrodon halys	0.100
Vipera berus berus	0.060
Echis carinatus	0.044
Bothrops jararaca	0.040
Agkistrodon saxatilis	0.028
Agkistrodon blomhoffii	0.020

[a] To 1 ml of substrate (0.5 mM ATEE in 0.05 M Tris–HCl, pH 8.5) 50 μl of venom (5 mg/ml) was added and the decrease in absorbance at 237 nm was recorded.

not degrade the fibrin plate. Some snake venom serine proteinases (thrombocytin, cerastocytin, serine proteinase PA-BJ) have platelet aggregating activity [33–35]. VLCTLP has neither aggregation-inducing nor inhibiting activity.

The ability of the purified enzyme to hydrolyse several types of peptides was examined. Masses of peptides and peptide fragments produced by VLCTLP hydrolysis were detected by MALDI-TOF mass spectrometry analysis. The oxidized insulin B-chain cleavage by VLCTLP is different from cleavages with other proteinases in venom. The enzyme catalysed the cleavage of Tyr^{16}–Leu^{17} and Tyr^{26}–Thr^{27} bonds in the oxidized insulin B-chain (Fig. 6A), Tyr^{10}–Ser^{11}, Tyr^{13}–Leu^{14} and Leu^{14}–Asp^{15} bonds in glucagon after overnight incubation and Tyr^{4}–Ile^{5} bond in angiotensins I and II. Remarkable cleavage of angiotensins was seen already after 5 min treatment (Fig. 6B).

We synthesized some tyrosine-containing peptides according to fibrinogen Aα-chain sequence (fragment 408–417) and pregnancy zone protein (PZP) sequence (fragment 687–693). The enzyme catalyses the cleavage of Tyr–His bond in the peptide fragment of fibrinogen ^{408}Glu-Tyr-His-Thr-Glu-Lys-Leu-Val-Thr-Ser^{417} and very slowly (after 20 h) Tyr–Val bond in the peptide fragment of PZP ^{687}Tyr-Val-Pro-Gln-Leu-Gly-Thr^{693}. Specificity studies against peptide substrates showed that VLCTLP catalysed the cleavage of peptide bonds preferably after tyrosine residues. It did not cleave chymotrypsin substrates Suc-Ala-Ala-Pro-Phe-pNA or Suc-Ala-Ala-Pro-Leu-pNA confirming the tyrosine specificity. VLCTLP is tyrosine-specific chymotrypsin-like enzyme.

3.3. Sequence of the cDNA encoding VLCTLP and the deduced amino acid sequence

The VLCTLP gene includes a 5′-UTR – nucleotides 1–179, a proenzyme coding region (nt 180–251), a mature enzyme coding region (nt 252–953), and a 3′-UTR (nt 954–1584). The full-length cDNA sequence of the VLCTLP encodes open reading frame of 257 amino acid residues that include a putative signal peptide of 18 amino acids, a proposed activation peptide of six amino acid residues (Gln-Lys-Ser-Ser-Glu-Leu) and mature serine proteinase of 233 amino acid residues (Fig. 7). VLCTLP contains twelve conserved cysteine residues that form six disulfide bonds.

The calculated molecular mass is 25580 that is considerably lower than the value obtained by MALDI-TOF MS (41.9 kDa). The difference could be explained with the heavy glycosylation of the enzyme. The deduced protein sequence exhibits 5 putative N-glycosylation sites, NXS/T, at the residues Asn44, Asn100, Asn116, Asn153 and Asn250 (Fig. 7) but all of them need not to be glycosylated. After deglycosylation the molecular mass of VLCTLP was reduced to about 27 kDa (Fig. 4). Sugars play important role in venom toxicology, not only increasing the solubility and stability of venom glycoproteins but also promoting their target

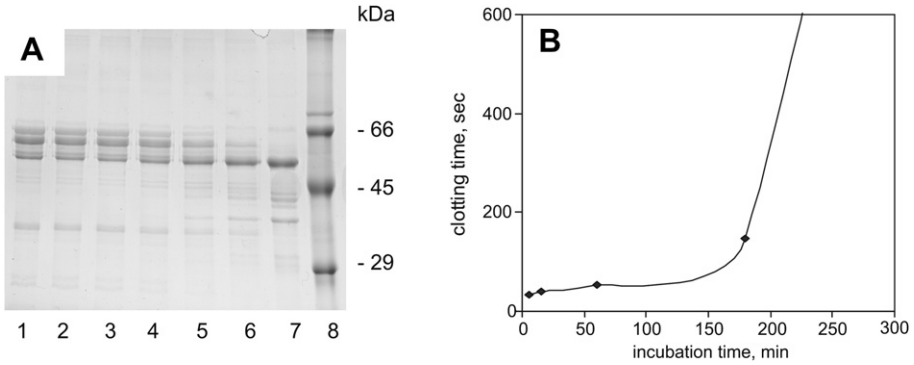

Fig. 5. Fibrinogenolytic activity. (A) 0.5 ml of 1% fibrinogen solution was incubated with 0.5 ml of enzyme (50 μg) at 37 °C in 0.05 M Tris–HCl buffer (pH 7.4), containing 0.1 M NaCl. At various time intervals 50 μl of the incubation mixture was withdrawn and added to 50 μl of denaturing solution (10 M urea, 4% SDS, 4% 2-mercaptoethanol). The samples were incubated overnight at 37 °C before electrophoresis. Specific cleavage of fibrinogen was shown on 10% SDS-polyacrylamide gel. 1 – fibrinogen; 2 – 5 min; 3 – 15 min; 4 – 60 min; 5 – 180 min; 6 – 420 min; 7 – 18 h; 8 – molecular mass markers. (B) 2 ml of 1% fibrinogen solution was incubated with 0.5 ml of enzyme (200 μg) at 37 °C in 0.05 M Tris–HCl buffer (pH 7.4), containing 0.1 M NaCl. At various time intervals 300 μl of the incubation mixture was withdrawn and 15 μl of thrombin (Sigma) was added. The fibrinogenolytic activity was determined indirectly as an increase in the clotting time.

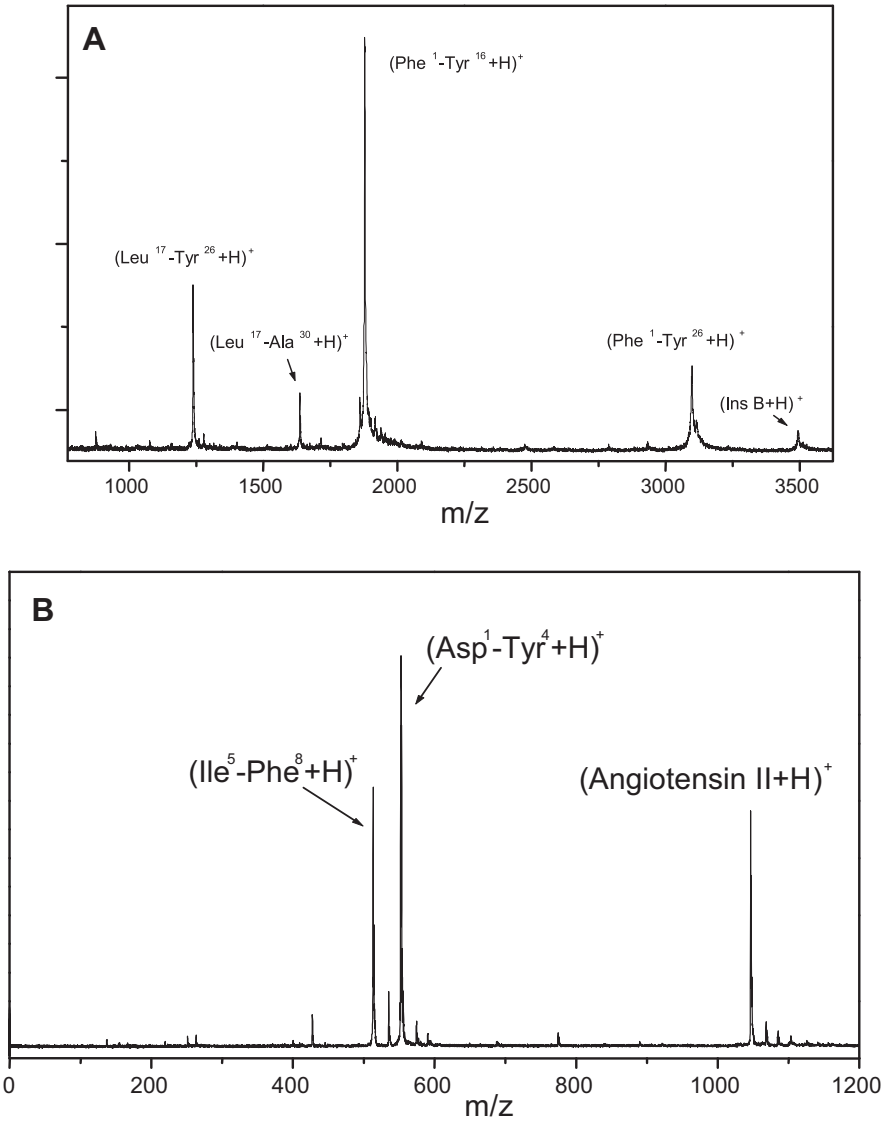

Fig. 6. MALDI-TOF mass-spectra of the cleavage products of peptides after treatment with VLCTLP. Matrix was 2,5-dihydroxybenzoic acid (DHB). (A) Oxidized insulin B-chain fragments after 3 h treatment. (B) Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) and cleavage fragments after 5 min treatment. Enzyme concentration 75 μg/ml.

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-179    ggcacgagtgtcagggttccagattgtgggccaccgagctgcttaatttgatcaaataaagt -118

-117    gctgcttgatcaagaagtctccgcttgggttatctgattagactgatacggtatctcagc   -58

 -57    tttacgtttcggactggaatcttacagggaaacagcttgccgtgcagagttgaagctATG     3
   1                                                               M     1
   4    GTGCTGATCAGAGTGCTAGCAAACCTTCTGCTACTACAGCTTTCTTATGCACAAAAGTCT    63
   2     V  L  I  R  V  L  A  N  L  L  L  L  Q  L  S  Y  A  Q  K  S     21
  64    TCTGAACTGGTCGTTGGAGGTGATGAATGTAACATAAATGAACATCGTTCCCTTGTATTC   123
  22     S  E  L  V  V  G  G  D  E  C  N  I  N  E  H  R  S  L  V  F     41
 124    TTGTATAACTCTAGCTTTGGCTGCGGTGGGACTTTGATCAACCAGCAATGGGTGCTCAGC   183
  42     L  Y  N  S  S  F  G  C  G  G  T  L  I  N  Q  Q  W  V  L  S     61
 184    GCTGCACACTGCGACATGGAAAATGTGCAGATATACCTTGGTTTGCATAACTTGCGTCTA   243
  62     A  A  H  C  D  M  E  N  V  Q  I  Y  L  G  L  H  N  L  R  L     81
 244    CGAAATCAGGATGAGCAGATAAGAGTTGCAGAGGAGAAGTTCTTTTGTCTCAGTAACAAA   303
  82     R  N  Q  D  E  Q  I  R  V  A  E  E  K  F  F  C  L  S  N  K    101
 304    AGCTATACCAAATGGGACAAGGACATCATGTTGATCAGGCTGAACAGTTCTGTTACCTAC   363
 102     S  Y  T  K  W  D  K  D  I  M  L  I  R  L  N  S  S  V  T  Y    121
 364    AATACACACATCGCGCCTCTCAGCTTGCCTTCCAGTCCTCCCCGTGTGGGCTCAGTTTGC   423
 122     N  T  H  I  A  P  L  S  L  P  S  S  P  P  R  V  G  S  V  C    141
 424    CGTATTATGGGATGGGGCGCAATCACATCTCCTAATGAGACTTTTCCCAATGTCCCCCAT   483
 142     R  I  M  G  W  G  A  I  T  S  P  N  E  T  F  P  N  V  P  H    161
 484    TGTGCTAACATCAACATACTCCGTTATTCAGTGTGTCGAGCAGCTTACAGAGGGTTACCG   543
 162     C  A  N  I  N  I  L  R  Y  S  V  C  R  A  A  Y  R  G  L  P    181
 544    GCACAAAGCAGAACACTGTGTGCAGGTATCCTGCAAGGAGGCATAGGTTCATGTATGGGT   603
 182     A  Q  S  R  T  L  C  A  G  I  L  Q  G  G  I  G  S  C  M  G    201
 604    GACTCTGGGGGACCGCTCATCTGTAATGGAGAAATCCAGGGCATTGTATCTTGGGGGGAC   663
 202     D  S  G  G  P  L  I  C  N  G  E  I  Q  G  I  V  S  W  G  D    221
 664    GATATTTGTGCCCAACCTCATAAGCCTGTCCACTACACCAAGGTCTTCGATTATAGTGAC   723
 222     D  I  C  A  Q  P  H  K  P  V  H  Y  T  K  V  F  D  Y  S  D    241
 724    TGGATACAGAGCATTATTGCAGGAAATACAACTGCAACTTGCCCCCTGTGAaaacttttg   783
 242     W  I  Q  S  I  I  A  G  N  T  T  A  T  C  P  L  stop          257
 784    aaaaatttaacaggaggaaatgtagcatattagtacatctcttctatatccctaatcata   843
 844    ttcaactgcattggaatatattcccaagcagtaaacttttaaagaatcaaataggactg   903
 904    cctttggagtaagaaatgctcaaaatagtgctgcagggatcatgtcccatttaatttcag   963
 964    tataaaacaatcttagtaaagtggaggtctgttttagggtgaggtgcaaagttttctgac  1023
1024    tctaaaatggacaattccaaatattttaacctctgaatatatttccatttctctccactt  1083
1084    ctgggacagtgggatccttgatgctctctgagcttgttttcttgcagacatttcattacc  1143
1144    cagctagttaacatcgccagtgctagaatattcttttctattggtactattgtggcattt  1203
1204    acaatacattcatgtggagtcatgcagtcaccacacaaacatatccatatactcgagtcc  1263
1264    cactgttgcttaaaaaggatcccagattaacccccacttcccaatcactaaattgaatct  1323
1324    tttgggaatcatactttaatgtaaattctcaggtatccacagcagtaaaatcatataaat  1383
1384    tgtcaaaaaaaaaaaaaaaaa                                         1405
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Fig. 7. Complete cDNA and deduced amino acid sequence of the VLCTLP precursor. The nucleotide sequence and the deduced amino acid sequence are numbered from the N-terminal amino acid of the preproprotein. The cDNA coding regions are shown in uppercase and 5′-and 3′-untranslated regions in lower-case letters. The N-terminus detected from protein sequence is in bold italics. Active site serine, histidine and aspartic acid residues are bold. The potential N-glycosylation sites are grey (Asn44; Asn100, Asn116, Asn153, Asn250). The peptide fragment sequences detected by LC-MS/MS are bold underlined. Tryptic peptide fragments detected by MALDI-TOF MS are underlined. Tryptic peptide fragments 115–136 and 143–169 were detected after deglycosylation of the enzyme.

recognition and specific binding in vivo [36]. VLCTLP is rather stable protein due to its 6 disulfide bonds and heavy glycosylation (5 putative glycosylation sites). It is well known that sugars increase the solubility and stability of venom glycoproteins.

3.4. Similarity of VLCTLP to other sequences

Fig. 8 shows the alignment of deduced amino acid sequences of the precursor of VLCTLP with several homologous sequences from different snake species.

The identity of protein sequences is highest between VLCTLP and VLBF, *V. lebetina* β-fibrinogenase, about 83%. Their first 32 N-terminal amino acid residues are completely identical (Fig. 8). The rate of identity with other 100 snake venom serine proteinases covers the range 63–76% (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These enzymes possess rather different substrate specificities including fibrinogenases, thrombin-like enzymes, plasminogen activators, factor V activators, protein C activators, kinin-releasing enzymes.

4. Discussion

Snake venom serine proteinases affecting haemostasis belong to a trypsin-like subfamily of proteinases that share a high degree of

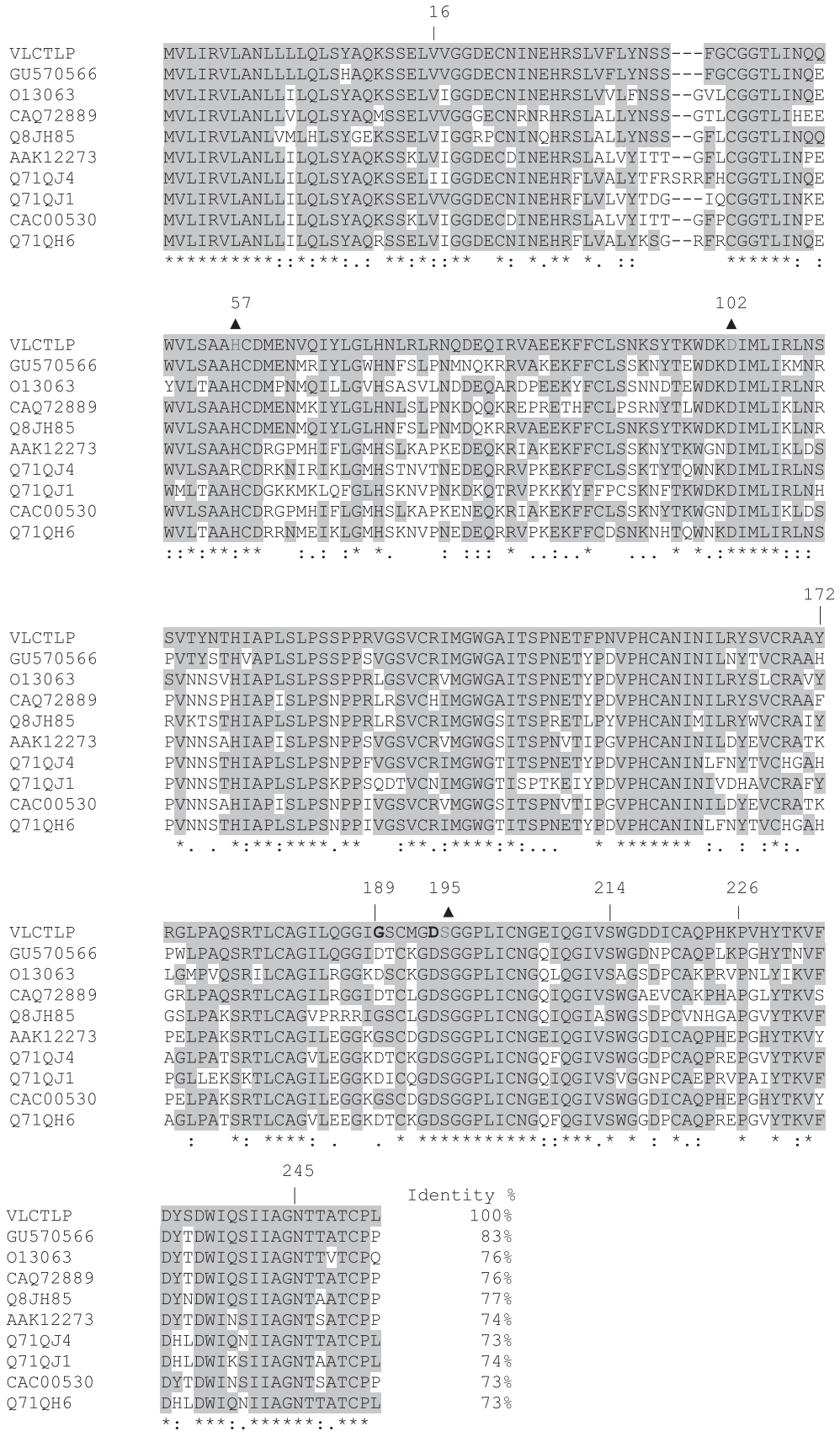

Fig. 8. Multiple sequence alignment of precursors of serine proteases from different snake venoms using CLUSTAL W [43]. VLCTLP-*Vipera Lebetina* chymotrypsin-like protease; GU570566-VLBF, *Vipera Lebetina* beta-fibrinogenase; O13063 – [*Trimeresurus gramineus*]; CAQ72889 – [*Echis ocellatus*]; Q8JH85 – serine alpha-fibrinogenase precursor [*Macrovipera lebetina*]; AAK12273 – thrombin-like enzyme precursor [*Deinagkistrodon acutus*]; Q71QJ4 – - serine protease KN4 precursor [*Viridovipera stejnegeri*], Q71QI1 – serine protease KN12 precursor [*Viridovipera stejnegeri*]; CAC00530 – acubin2 [*Deinagkistrodon acutus*]; Q71QH6 – serine protease KN13 precursor [*Viridovipera stejnegeri*]. Closed triangles indicate the amino acid residues (His57, Asp102, Ser195, Chymotrypsinogen numbering.) involved in the catalytic reaction. "*" indicates positions which have a single, fully conserved residue; ":"indicates that one of the 'strong' amino acid groups is fully conserved; "." indicates that one of the 'weaker' groups is fully conserved.

sequence identity despite exhibiting remarkably high substrate selectivity. They are composed of approximately 235 amino acids and contain 12 conserved cysteine residues paired in six disulfide bridges. The *V. lebetina* venom contains several serine proteases, most of them are specific enzymes: Factor V activator, bradykinin-releasing enzyme, β-fibrinogenase – these enzymes catalyse the hydrolysis of BAEE. The venom contains also two serine proteinases that do not catalyse the hydrolysis of BAEE: α-fibrinogenase (VLAF) [15] and VLCTLP. We have shown that VLAF effectively hydrolyses casein, VLCTLP very weakly catalyses the cleavage of azocasein but differently from other snake venom serine proteinases VLCTLP has ATEE-hydrolysing activity.

The proteolytic specificity is usually characterized by the hydrolysis of oxidized insulin B-chain. Differently from arginine esterases that catalyse mainly the hydrolysis after Arg residue, VLCTLP cleaves bonds after Tyr residues.

The molecule of VLCTLP contains the amino acids forming catalytic triad His57, Asp102 and Ser195 (Fig. 8) (chymotrypsinogen numbering) like all enzymes belonging to the clan SA [37]. The sequences around those amino acid residues were found to be highly homologous. The positions of 12 half-cystines are identical among all the sequences, suggesting that the proteins encoded will take similar tertiary structure. On the basis of the evolutionary markers VLCTLP is the member of the S1 (chymotrypsin) subfamily [38]. Ser195 and Ser214 are both encoded by TCT, and the position 226 is occupied by proline (Fig. 8). In almost all of the active serine proteinases, the N-terminal residue (Val16, Fig. 8) forms an internal salt bridge between its amino group and the side chain carboxyl group of Asp194 (Fig. 8) [39]. Apart from Asp102 and Asp194 (Fig. 8) there is the third essential aspartate in the molecule of trypsin-like serine proteinases – Asp189 that is located in the bottom of the primary specificity pocket near the active site and forms a salt bridge with the basic residue of the scissile bond [40]. Asp189, six residues before the catalytic amino acid Ser195 residue, is conserved in many snake venom serine proteinases, indicating that it is responsible for the trypsin-like specificity. Substitution of Asp189 resulted in 10^5 – fold decrease toward P1 Arg/Lys substrate [41]. In the case of VLCTLP Asp189 is replaced by Gly189 (Fig. 8) that points to the possible lack of the trypsin-like substrate specificity for basic amino acids at the P1 position.

In summary, some mutations in the primary structure may essentially change the specificity of the enzyme as it happened in the case of VLCTLP. The point mutation in codon for Asp189 (A–G) changes it to codon for Gly (Fig. 7, 589G) and deprives it from the arginine esterase activity. The chymotrypsin-like activity is difficult to explain. Hedstrom et al. [42] found that Tyr172 residue might be a specificity determinant because the trypsin Tyr172Trp mutant showed 2–15% of chymotrypsin activity. The Tyr172 of VLCTLP is conserved, so there must be some other reason for the chymotrypsin-like specificity.

Thus, snake venom serine proteinases share a high degree of sequence identity (60–80%) and show at the same time a high substrate specificity. We have found a novel serine proteinase in *V. lebetina* venom catalysing the hydrolysis of ATEE. Specificity studies against tyrosine and phenylalanine residues containing peptide substrates showed that VLCTLP catalysed the cleavage of peptide bonds after tyrosine residues.

The best substrates for VLCTLP were angiotensins I and II. Angiotensin II is an octapeptide that causes vasoconstriction, increased blood pressure, and release of aldosterone from the adrenal cortex. Proteinases involved in processing of angiotensin peptides have been collectively termed “angiotensinases”. Angiotensinases are comprised of three groups of peptidases: amino-, endo- and carboxypeptidases [44]. Similarly to angiotensinase B (chymotrypsin-like endopeptidase) [45], VLCTLP acts on the bond between residues 4 and 5 in angiotensin II to release an N-terminal aspartyl tetrapeptide Ang-(1–4) and a C-terminal tetrapeptide Ang-(5–8), destroying the biological activity of angiotensin II.

VLCTLP is unique among known snake venom serine proteases for its substrate specificity. To the best of our knowledge VLCTLP is the only enzyme isolated from snake venoms that catalyses the hydrolysis of the N-acetyl-L-tyrosine ethyl ester (ATEE).

Conflict of interest

The authors declare there are no conflicts of interest.

Acknowledgements

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References

[1] S. Braud, C. Bon, A. Wisner, Snake venom proteins acting on hemostasis, Biochimie 82 (2000) 851–859 Review.
[2] T. Matsui, Y. Fujimura, K. Titani, Snake venom proteases affecting hemostasis and thrombosis, Biochim. Biophys. Acta 1477 (2000) 146–156 Review.
[3] R.M. Kini, Serine proteases affecting blood coagulation and fibrinolysis from snake venoms, Pathophysiol. Haemost. Thromb. 34 (2005a) 200–204 Review.
[4] R.M. Kini, The intriguing world of prothrombin activators from snake venom, Toxicon 45 (2005b) 1133–1145 Review.
[5] R.M. Kini, Anticoagulant proteins from snake venoms: structure, function and mechanism, Biochem. J. 397 (2006) 377–387.
[6] S. Swenson, F.S. Markland Jr., Snake venom fibrin(ogen)olytic enzymes, Toxicon 45 (2005) 1021–1039.
[7] A. Bazaa, N. Marrakchi, M. El Ayeb, L. Sanz, J.J. Calvete, Snake venomics: comparative analysis of the venom proteomes of the Tunisian snakes *Cerastes cerastes*, *Cerastes vipera* and *Macrovipera lebetina*, Proteomics 5 (2005) 4223–4235.
[8] J.J. Calvete, C. Marcinkiewicz, L. Sanz, Snake venomics of *Bitis gabonica gabonica*. Protein family composition, subunit organization of venom toxins, and characterization of dimeric disintegrins bitisgabonin-1 and bitisgabonin-2, J. Proteome Res. 6 (2007) 326–336.
[9] J.J. Calvete, E. Fasoli, L. Sanz, E. Boschetti, P.G. Righetti, Exploring the venom proteome of the western diamondback rattlesnake, *Crotalus atrox*, via snake venomics and combinatorial peptide ligand library approaches, J. Proteome Res. 8 (2009) 3055–3067.
[10] J.W. Fox, S.M. Serrano, Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases, Toxicon 45 (2005) 969–985.
[11] S.M. Serrano, R.C. Maroun, Snake venom serine proteinases: sequence homology vs. substrate specificity, a paradox to be solved, Toxicon 45 (2005) 1115–1132 Review.
[12] J. Siigur, K. Tonismagi, K. Trummal, A. Aaspollu, M. Samel, H. Vija, J. Subbi, N. Kalkkinen, E. Siigur, *Vipera lebetina* venom contains all types of snake venom metalloproteases, Pathophysiol. Haemost. Thromb. 34 (2005) 209–214.
[13] E. Siigur, M. Samel, K. Tõnismägi, J. Subbi, T. Reintamm, J. Siigur, Isolation, properties and N-terminal amino acid sequence of a factor V activator from *Vipera lebetina* (Levantine viper) snake venom, Biochim. Biophy. Acta 1429 (1998) 239–248.
[14] E. Siigur, A. Aaspõllu, J. Siigur, 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 (1999) 328–332.
[15] M. Samel, J. Subbi, J. Siigur, E. Siigur, Biochemical characterization of fibrinogenolytic serine proteinases from *Vipera lebetina* snake venom, Toxicon 40 (2002) 51–54.
[16] A.T. Tu, A. Chua, G.P. James, Proteolytic enzyme activities in a variety of snake venoms, Toxicol. Appl. Pharmacol. 8 (1966) 218–223.
[17] G.W. Schwert, Y. Takenaka, A spectrophotometric determination of trypsin and chymotrypsin, Biochim. Biophys. Acta 16 (1955) 570–575.
[18] J. Siigur, M. Samel, K. Tõnismägi, J. Subbi, E. Siigur, A.T. Tu, Biochemical characterization of lebetase, a direct-acting fibrinolytic enzyme from *Vipera lebetina* snake venom, Thromb. Res. 90 (1998) 39–49.
[19] T. Astrup, S. Müllertz, The fibrin plate method for estimating of fibrinolytic activity, Archs. Biochem. Biophys. 40 (1952) 346–351.
[20] A. Bee, R.D.G. Theakston, R.A. Harrison, S.D. Carter, Novel in vitro assays for assessing the haemorrhagic activity of snake venoms and for demonstration of venom metalloproteinase inhibitors, Toxicon 39 (2001) 1429–1434.
[21] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[22] O. Vesterberg, Isoelectric focusing of proteins in polyacrylamide gels, Biochim. Biophys. Acta 257 (1972) 11–19.

[23] E. Siigur, A. Aaspollu, K. Trummal, K. Tõnismagi, I. Tammiste, N. Kalkkinen, J. Siigur, Factor X activator from *Vipera lebetina* venom is synthesized from different genes, Biochim. Biophys. Acta 1702 (2004) 41–51.
[24] M. Poutanen, L. Salusjärvi, L. Ruohonen, M. Penttilä, N. Kalkkinen, Use of matrix-assisted laser desorption/ionization time-of-flight mass mapping and nanospray liquid chromatography/electrospray ionization tandem mass spectrometry sequence tag analysis for high sensitivity identification of yeast proteins separated by two-dimensional gel electrophoresis, Rapid Commun. Mass Spectrom. 15 (2001) 1685–1692.
[25] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.
[26] E. Siigur, A. Aaspollu, A.T. Tu, J. Siigur, cDNA cloning and deduced amino acid sequence of fibrinolytic enzyme (lebetase) from *Vipera lebetina* snake venom, Biochem. Biophys. Res. Commun. 224 (1996) 229–236.
[27] K. Tõnismägi, M. Samel, K. Trummal, G. Ronnholm, J. Siigur, N. Kalkkinen, E. Siigur, L-amino acid oxidase from *Vipera lebetina* venom: isolation, characterization, effects on platelets and bacteria, Toxicon 48 (2006) 227–237.
[28] K. Trummal, K. Tõnismägi, E. Siigur, A. Aaspõllu, A. Lopp, T. Sillat, R. Saat, L. Kasak, I. Tammiste, P. Kogerman, N. Kalkkinen, J. Siigur, A novel metalloprotease from *Vipera lebetina* venom induces human endothelial cell apoptosis, Toxicon 46 (2005) 46–61.
[29] H. Vija, M. Samel, E. Siigur, A. Aaspõllu, K. Trummal, K. Tõnismägi, J. Subbi, J. Siigur, Purification, characterization, and cDNA cloning of acidic platelet aggregation inhibiting phospholipases A(2) from the snake venom of *Vipera lebetina* (Levantine viper), Toxicon 54 (2009) 429–439.
[30] V. Paalme, K. Trummal, M. Samel, K. Tõnismägi, L. Järvekülg, H. Vija, J. Subbi, J. Siigur, E. Siigur, Nerve growth factor from *Vipera lebetina* venom, Toxicon 54 (2009) 329–336.
[31] H. Vija, M. Samel, E. Siigur, A. Aaspõllu, K. Tõnismägi, K. Trummal, J. Subbi, J. Siigur, VGD and MLD-motifs containing heterodimeric disintegrin viplebedin-2 from *Vipera lebetina* snake venom. Purification and cDNA cloning, Comp. Biochem. Physiol. B. 153 (2009) 253–260.
[32] E. Siigur, J. Siigur, Purification and characterization of lebetase, a fibrinolytic enzyme from *Vipera lebetina* (snake) venom, Biochim. Biophys. Acta 1074 (1991) 223–229.
[33] E.P. Kirby, S. Niewiarowski, K. Stocker, C. Kettner, E. Shaw, T.M. Brudzynski, Thrombocytin, a serine protease from *Bothrops atrox* venom. 1. Purification and characterization of the enzyme, Biochemistry 18 (1979) 3564–3570.
[34] N. Marrakchi, R.B. Zingali, H. Karoui, C. Bon, M. el Ayeb, Cerastocytin, a new thrombin-like platelet activator from the venom of the Tunisian viper *Cerastes cerastes*, Biochim. Biophys. Acta 1244 (1995) 147–156.
[35] S.M. Serrano, R. Mentele, C.A. Sampaio, E. Fink, Purification, characterization, and amino acid sequence of a serine proteinase, PA-BJ, with platelet-aggregating activity from the venom of *Bothrops jararaca*, Biochemistry 34 (1995) 7186–7193.
[36] H.S. Chen, J.M. Chen, C.W. Lin, K.H. Khoo, I.H. Tsai, New insights into the functions and N-glycan structures of factor X activator from Russell's viper venom, FEBS J. 275 (2008) 3944–3958.
[37] A.J. Barrett, N.D. Rawlings, Families and clans of serine peptidases, Arch. Biochem. Biophys. 318 (1995) 247–250.
[38] M.M. Krem, E. Di Cera, Molecular markers of serine protease evolution, EMBO J. 20 (2001) 3036–3045.
[39] D. Wang, W. Bode, R. Huber, Bovine chymotrypsinogen A. X-ray crystal structure analysis and refinement of a new crystal form at 1.8 Å resolution, J. Mol. Biol. 185 (1985) 595–624.
[40] J. Janin, C. Chothia, Stability and specificity of protein-protein interactions: the case of the trypsin–trypsin inhibitor complexes, J. Mol. Biol. 100 (1976) 197–211.
[41] H. Czapinska, J. Otlewski, Structural and energetic determinants of the S1-site specificity of serine proteinases, Eur. J. Biochem. 260 (1999) 571–595.
[42] L. Hedstrom, J.J. Perona, W.J. Rutter, Converting trypsin to chymotrypsin: residue 172 is a substrate specificity determinant, Biochemistry 33 (1994) 8757–8763.
[43] J.D. Thompson, D.J. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucl. Acids Res. 22 (1994) 4673–4680.
[44] V.T. Karamyan, R.C. Speth, Enzymatic pathways of the brain renin-angiotensin system: unsolved problems and continuing challenges, Regul. Peptides 143 (2007) 15–27.
[45] P.A. Khairallah, I.H. Page, Plasma angiotensinases, Biochem. Med. 1 (1967) 1–8.