BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **224,** 229–236 (1996)
ARTICLE NO. 1012

cDNA Cloning and Deduced Amino Acid Sequence of Fibrinolytic Enzyme (Lebetase) from *Vipera lebetina* Snake Venom[1]

Ene Siigur, Anu Aaspõllu, Anthony T. Tu,* and Jüri Siigur

*Institute of Chemical Physics and Biophysics, Tallinn, EE0026, Estonia; and *Colorado State University, Department of Biochemistry and Molecular Biology, Fort Collins, Colorado 80523*

Received May 29, 1996

The complete amino acid sequence of lebetase is deduced from the nucleotide sequence of a cDNA clone isolated by screening a venomous gland c DNA library of Central Asian *Vipera lebetina* snake. The cDNA sequence with 2011 basepairs encodes an open reading frame of 478 amino acids which includes an 18 amino acid signal peptide, plus an 175 amino acid segment of zymogen-like propeptide, a mature protein of 204 amino acids, a spacer of 18 amino acids and a disintegrin-like peptide of 63 amino acids. The mature protein lebetase as isolated from the crude venom has the molecular weight of approximately 23.7 kD and, thus, lebetase as well as several other snake venom metalloproteinases is translated as a precursor protein, which may be processed posttranslationally. The lebetase proprotein has a ''cysteine switch'' motif **(PKMCGV)** similar to that involved in the activation of matrix metalloproteinase zymogens. The mature protein (residues 223-427) shows the strongest similarity with fibrolase(63% identity), fibrinolytic enzyme from *Agkistrodon contortrix contortrix* venom. The metalloproteinase domain has a typical zinc-chelating sequence **(HEXXHXXGXXH).** In the disintegrin-like domain of protein, the **RGD** sequence is replaced by **VGD.**

Fibrin(ogen)olytic enzymes have been described from different snake venoms including members of Crotalidae, Viperidae and Elapidae families (for reviews, see 1,2). Fibrin(ogen)-olytic enzymes from snake venoms directly split off fragments of α-, β- and γ-chains of fibrinogen. There are mainly two classes of fibrin(ogen)olytic enzymes: 1)$\alpha(\beta)$-fibrinogenases are zinc-metalloproteinases, which catalyse also fibrin hydrolysis, 2)β-fibrinogenases are thermostable serine proteinases which split the β-chain of fibrinogen. Recently a novel plasminogen activator was found in snake venom (3) and it acts as indirect fibrinolysin.

The study of the venom from the Levantine viper *Vipera lebetina* has demonstrated the existence of different proteinases in the venom. We showed that the venom of *Vipera lebetina* contains both coagulant and anticoagulant enzymes: factor X activator, β- and $\alpha(\beta)$-fibrin-(ogen)olytic enzymes (4-7). The $\alpha(\beta)$-fibrin(ogen)olytic enzyme, named lebetase, from *Vipera lebetina* venom cleaves α-chain and more slowly β-chain in fibrinogen and in fibrin, without activating plasminogen(6). Many of fibrin(ogen)olytic metalloproteinases have been shown to possess different activities: caseinolytic, fibrinolytic, hemorrhagic. Such a functional diversity within this group of similar proteinases raises questions of the relationship between their structure and their effect. It is not clear what structural features are responsible for hemorrhagic activity of snake venom metalloproteinases. It has been demonstrated that some of the purified fibrinolytic enzymes (fibrolase, atroxase) are nonhemorrhagic (8,9). Lebetase has very low hemorrhagic activity.

In the present work we determined the nucleotide sequence of a fibrin(ogen)olytic metalloproteinase from *Vipera lebetina* venom by cDNA cloning and sequencing. From the deduced

[1] The sequence data of *Vipera lebetina* mRNA for fibrinolytic metalloproteinase have been deposited in the EMBL Nucleotide Sequence Database under Accession No. X97894.

0006-291X/96 $18.00

amino acid sequence we found that the metalloproteinase is synthesized with disintegrin-like part which most probably is cleaved off posttranslationally by proteinase. This is the first sequence of fibrinolytic enzyme from Viperidae venoms. Fibrinolytic enzymes are under investigation for potential applications as thrombolytic agents.

MATERIALS AND METHODS

Protein preparation. Lebetase was purified from crude *V. lebetina* venom (Tashkent Serpentarium, Uzbekistan) using the purification scheme of Siigur and Siigur (6).Fibrinolytic activity was determined by fibrin-plate method of Astrup and Müllertz (10).

Cyanogen bromide digestion of lebetase. Lebetase (1.0 mg) was dissolved in 300 μl of solution containing 70% of trifluoroacetic acid and 30 mg/ml CNBr. The mixture was incubated for 18 h at room t^0 in the dark. The reaction was stopped by adding 10 volumes of water, freezed and lyophilized thrice. The BrCN-fragments were separated on 10-20% Tricine-SDS-PAGE and electrotransferred onto PVDF-membrane. One cleavage fragment was excised for NH_2-terminal amino acid sequence.

Protein sequencing. Protein sequencing was performed by Edman degradation on an Applied Biosystems ABI 173A Sequenator.

Poly(A)$^+$ RNA isolation and cDNA library construction. mRNA was isolated from the venom glands of one adult Central Asian *Vipera lebetina* snake three days after milking using mRNA isolation kit (Stratagene, La Jolla, USA). Poly(A)$^+$rich RNA was prepared using oligo(dT)-cellulose column. The total yield of poly(A)$^+$RNA was about 25 μg. A cDNA library was constructed in the Uni-ZAP XR vector(Stratagene, La Jolla, USA). Double-stranded cDNA was synthesized from 10 μg of poly(A)$^+$RNA preparation, cloned into Stratagene's Uni-ZAP XR vector and packaged (Gigapack II Gold Packaging Extract, Stratagene) according to the instructions of the manufacturer. The packaged DNA was titered and found to contain 5.5×10^6 recombinants. The packaged library was plated on *Escherichia coli* XL1-BLUE MRF′ strain and amplified as a plate lysate on NZY agar plates.

Design and synthesis of primers for cDNA amplification. On the basis of the determined internal peptide sequence of lebetase (N-terminal of lebetase is blocked) and the highly conserved domains sequence of analogous metalloproteinases, and the analysis of genetic code usage for the venom metalloprotease cDNA sequences, two oligonucleotide primers, P_1 and P_2, were designed for the amplification of a cDNA internal fragment by PCR: Primer P_1 (22 residues): **(5′-GGTCACGAAATACGATGGTGAT3′)** is oriented in the sense direction and corresponds to the internal sequence residues 20-27 of lebetase. Primer P_2 (26 residues): **(5′-GCCGTGAGTAACTGGGCATTATCATG-3′)** is oriented in the antisense direction and corresponds to conserved metalloproteinase sequence 94-102. Oligonucleotide primers were synthesized in an Applied Biosystems 381DNA synthesizer and purified on C18 column. The double-stranded cDNA synthesized from the mRNA from *V.lebetina* venom gland was used as a template. The amplification by PCR with the primers P_1 and P_2 was carried out in a Perkin Elmer Thermal Cycler using *Taq* DNA polymerase (Promega, USA), under the following conditions: denaturation (1 min, at 94°C), annealing (1 min, at 47°C) and extension (1 min 30 sec, at 72°C) −30 cycles were performed. The PCR-reaction product was analyzed by 1% agarose gel electrophoresis and purified by means of Magic PCR Preps DNA Purification Resin (Promega, USA). The purified DNA was ligated into pUC57/T vector (''Fermentas'', Lithuania). Recombinant plasmids containing PCR fragment insert were sequenced using USB Sequenase Version 2.0 Sequencing Kit. The PCR product was ^{32}P-labeled by random priming with Klenow fragment (Promega, USA) and [α-^{32}P] ATP (Amersham, UK) and used to probe the cDNA library.

cDNA library screening. The library was plated on *E. coli* XL1-BLUE MRF′ and grown overnight at 37°C in NZY plates. The plates were then chilled for 2 h, and plaques were lifted onto nylon membranes (Hybond-N, Amersham, UK) and processed as recommended by manufacturer. After baking for 2 h at 80°C filters were prehybridized for 2 h at 42°C. Labeled probe was used at a concentration of 5×10^5 cpm/ml. The hybridization was allowed to proceed overnight at 42°C. After hybridization, the filters were washed under high stringency conditions and exposed to X-ray film (Amersham Hyperfilm TM) at −70°C.

Analysis of the positive clones. The positive plaques were cut off and purified by secondary screening. For excision of the phagemid, the XL1-BLUE MRF′ cells were coinfected with an aliquot of the phage of interest and the ExAssist/SOLR system helper phage. The excised phagemid was plated in freshly grown XL1-BLUE MRF′ cells in LB-ampicillin plates (50 μg/ml) and allowed to grow overnight at 37°C. Plasmid DNA was isolated by alkaline miniprep purification, restriction-digested with NotI and XhoI for 2 h at 37°C to remove the insert and analyzed using 1% agarose gel electrophoresis. The isolate with the highest molecular weight was subjected to sequencing.

DNA sequencing. The DNA was restriction-digested with several restriction enzymes. Isolated DNA fragments were subcloned into p-Bluescript and sequenced along both strands (Fig. 1). From the overlapping zones, the sequence of the full-length cDNA was constructed. DNA sequencing was performed manually by dideoxynucleotide chain termination method (11) using USB Sequenase Version 2.0 sequencing kit.

Sequence analysis. The cDNA sequences and deduced amino acid sequences were compared to sequences in the

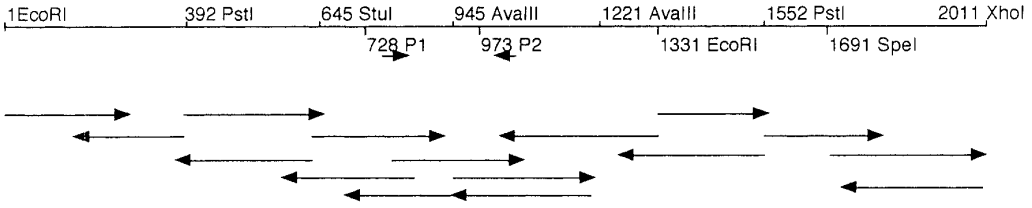

FIG. 1. Partial restriction map and sequencing strategy used to obtain the nucleotide sequence of the cDNA clone Le3. The directions of sequence determinations are shown by horizontal arrows. P1 and P2 are the primers for PCR.

Gen Bank and PIR databases, using the FASTA program (12). Sequence manipulations, translations and comparisons were performed using PCGENE software (Fig.2 and 3).

RESULTS AND DISCUSSION

Isolation of Lebetase

Lebetase was purified from crude *V.lebetina* venom using the purification scheme of Siigur and Siigur (6). Homogeneity was established by SDS-PAGE. Lebetase is a single chain Aα, Bβ-fibrinogenase. Fibrinolytic activity of lebetase was about 20-times higher than that of the crude venom. Lebetase showed no hemorrhagic activity up to 50 μg of protein.

Sequence of Lebetase

Direct sequencing of lebetase resulted in no sequence, indicating that the Edman degradation was blocked. Six peptide fragments were obtained after lebetase was cleaved with CNBr. The sequence of the bottom band of gradient electrophoresis was **-VTKYDGDLAAIRTWAHQL-VNNIN-.** On the basis of the sequence of a CNBr-peptide the oligonucleotide primer 1 was synthesized for PCR reaction. The primer 2 was synthesized on the basis of conserved region of metalloproteinases (see Fig. 1)

The sequence of the 246-bp PCR product of cDNA amplification using previously mentioned primers was found to have high similarity (~70% of identity) with members of the snake venom metalloproteinase gene family. The 246 bp PCR product was used to screen the cDNA library made with the mRNA from *V.lebetina* venom glands. The clone (Le3) which was selected to be completely sequenced consisted of 2011 base pairs. It has an open reading frame of 1434 bp, predicting a zymogen form of 478 amino acids.

Figure 2 shows the cDNA sequence of lebetase and translated protein sequence from the initiation codon to the termination codon. The deduced amino acid sequence from this open reading frame is 478 amino acids, much larger than would be necessary to encode a 23.7 kDa protein. We assume the presence of a proenzyme region with the conserved signal peptide(193 amino acids), a zinc metalloproteinase region(204 amino acids), and a carboxyterminal peptide (81 amino acid), which must be posttranslationally removed. A conserved **PKMCGV** sequence located between the signal sequence and the mature protein has been identified in the encoded sequence of clone Le3, as well as in the other propeptides of snake venom metalloproteinases and matrix metalloproteinases(25). The function of this region is to bind to the active-site zinc via the cysteinyl sulfur and prevent proteolytic activity of zymogen (17).

Similarity of Lebetase to Other Sequences

When the nucleotide sequence of the lebetase clone (Le3- 2011 bp) was compared with the 1794 bp and 1771 bp cDNA sequences for metalloproteinases from *A. c. laticinctus* (13), 85.7% and 84.1% identities were discovered. Comparison of the translated open reading frames of these clones showed 74.0% and 70.4% identity over 415 amino acid overlap.

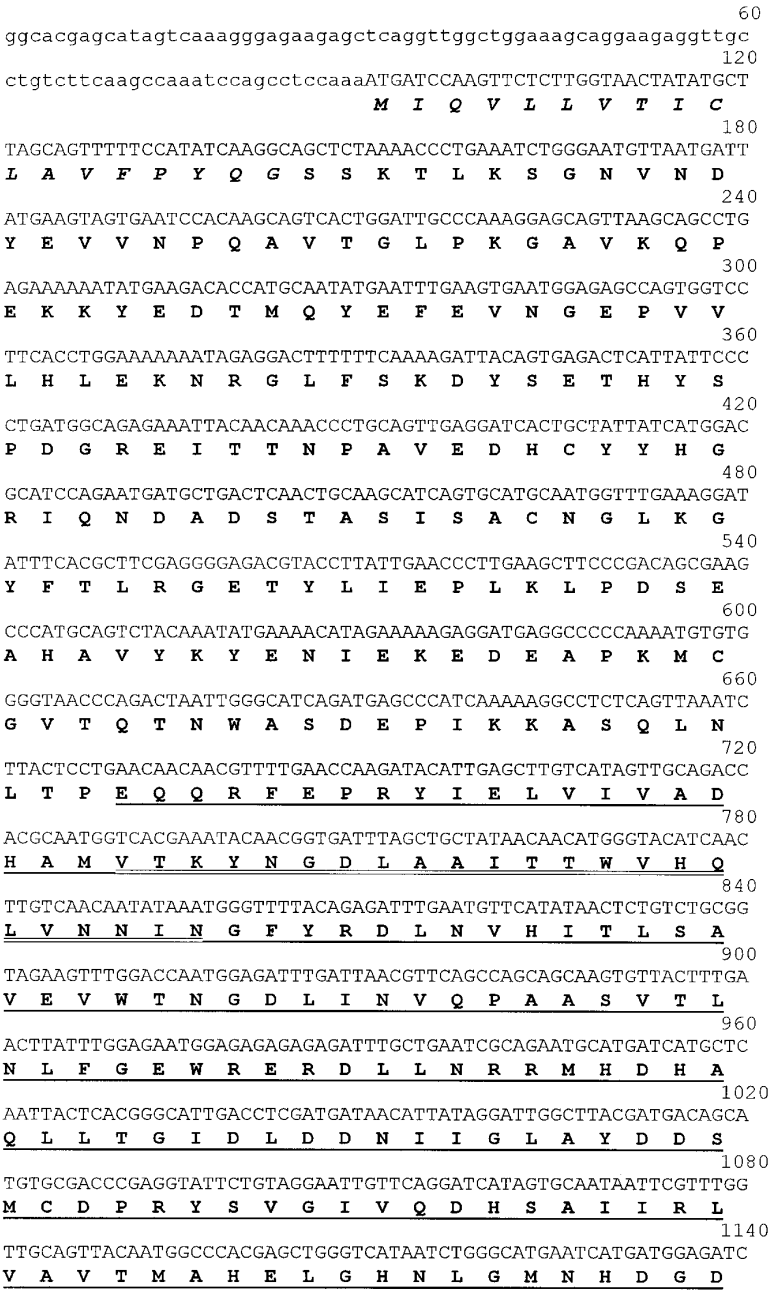

FIG. 2. Nucleotide and deduced amino acid sequences of the precursor for fibrinolytic protease from one cDNA clone (Le3). The nucleotide sequence of 2011 base pairs is shown above the amino acid sequence of 478 residues, which includes an 18 amino acid signal peptide plus an 175 amino acid propeptide, an 204 amino acid mature peptide, 18 amino acid spacer peptide and a disintegrin-like peptide of 63 amino acids. Nucleotide sequences are numbered in every 60 nucleotides. Amino acids are denoted by one-letter symbols. The signal peptide is in italics, the sequence of mature protein is underlined. The 23 amino acid residues, which were also sequenced directly from the peptide fragment of lebetase after BrCN cleavage, are doubly underlined. 5′UTR and 3′UTR sequences are shown in lowercase letters. A polyadenylation signal (aataaa) is dotted.

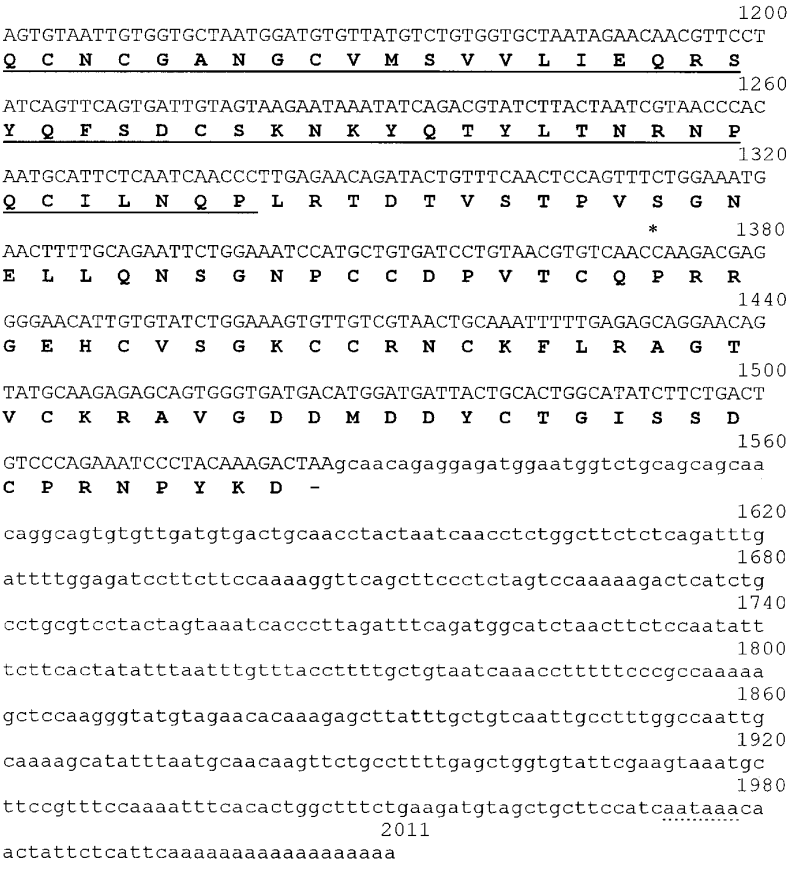

FIG. 2—*Continued*

Comparison of the deduced mature protein sequence to the Blitz EBI database confirmed a strong similarity to other snake venom metalloproteinases, especially for fibrinolytic metalloproteinases (fibrolase 63%, atroxase 59.8% but also for hemorrhagic and nonhemorrhagic proteinases (50-60%) as seen in Fig. 3.

The deduced amino acid sequence of lebetase has the zinc-binding motif **H-E-X-X-H-X-X-G-X-X-H** in the catalytic domain, which is conserved in the snake venom metalloproteinases (14-18). Lebetase belongs to the reprolysin family of metalloproteinases, including nonhemorrhagic and hemorrhagic proteinases and a number of mammalian reproductive proteins. In this family the third histidine is followed by a conserved aspartic acid (Fig. 3). (24)

The highly conserved cysteine residues allow the prediction of the same pattern of disulfide bonds for lebetase (C_{119}-C_{199}, C_{159}-C_{183}, C_{161}-C_{166}, numbering according to lebetase) as determined for fibrolase (8,18). These results indicate that lebetase belongs to the three-disulfide bond proteinase class. The majority of the reprolysins are three-disulfide proteinases (14). Fibrinolytic enzyme atroxase (9), nonhemorrhagic adamalysin II (15, 16) and hemorrhagic atrolysin C (17) have two disulfide bonds. According to Fox& Bjarnason (17) the position 408 (numbering in this paper*, Fig. 2) is critical for the maintenance of the protein structure. If there is a cysteine residue in this position, it will form a disulfide bond to free cysteine in the succeeding disintegrin-like domain, processing

```
                10         20         30         40         50
LEBE_   <EQQRFEPRYI ELVIVADHAM VTKYNGDLAA ITTWVHQLVN NINGFYRDLN
ATRO_   EDQQNLSQRYI ELVVVADHRV FMKYNSDLNI IRKRVHELVN TINGFYRSLN
FIBR_     <EQRFPQRYV QLVIVADHRM NTKYNGDSDK IRQWVHQIVN TINEIYRPLN
ADAM_   <EQNLPQRYI ELVVVADRRV FMKYNSDLNI IRTRVHEIVN IINGFYRSLN
HR1B_   <EQRFPRRYI KLAIVVDHGI VTKHHGNLKK IRKWIYQLVN TINNIYRSLN
HR2A_   <EQRFPQRYI ELAIVVDHGM YTKYSSNFKK IRKRVHQMVN NINEMYRPLN
HR2_T     ERFPQRYI ELAIVVDHGM YKKYNQNSDK IKVRVHQMVN HINEMYRPLN
HRTD_   <EQNLPQRYI ELVVVADHRV FMKYNSDLNT IRTRVHEIVN FINGFYRSLN
HRTE_     NTEHQRYV ELFIVVDHGM YTKYNGDSDK IRQRVHQMVN IMKESYTYMY
ACLPF   EQQGFPQRYV ELVIVADHRM NTKYNGDSDK IRQWVHQIVN TINEIYRPLN
ACLPH     NYQYQRYV ELVTVVDHGM YTKYNGDSDK IRQWVHQMVN TMKESYRYMY
                60         70         80         90        100
LEBE_   VHITLSAVEV WTNGDLINVQ PAASVTLNLF GEWRERDLLN RRMHDHAQLL
ATRO_   IDVSLTDLEI WSDQDFITVQ SSAKNTLNSF GEWREADLLR RKSHDHAQLL
FIBR_   IQFTLVGLEI WSNQDLITVT SVSHDTLASF GNWRETDLLR RQRHDNAQLL
ADAM_   IDVSLVNLEI WSGQDPLTIQ SSSSNTLNSE GLWREKVLLN KKKKDNAQLL
HR1B_   ILVALVYLEI WSKQNKITVQ SASNVTLDLF GDWRESVLLK QRSHDCAQLL
HR2A_   IAITLSLLDV WSEKDLITMQ AVAPTTARLF GDWRETVLLK QKDHDHAQLL
HR2_T   IAISLNRLQI WSKKDLITVK SASNVTLESF GNWRETVLLK QQNNDCAHLL
HRTD_   IHVSLTDLEI WSNEDQINIQ SASSDTLNAF AEWRETDLLN RKSHDNAQLL
HRTE_   IDILLAGIEI WSNGDLINVQ PASPNTLNSF GEWRETDLLK RKSHDNAQLL
ACLPF   IRFALVGLEI WSNQDLITVT SVSHDTLASF GNWRETDLLR RQRHDNAQLL
ACLPH   IDISLAGVEI WSNKDLIDVQ PAARHTLDSF GEWRERDLLH RISHDNAQLL
                110        120        130        140        150
LEBE_   TGIDLDDNII GLAYDDSMCD PRYSVGIVQD HSAIIRLVAV TMA**HELGHNL**
ATRO_   TAINFEGKII GRAYTSSMCN PRKSVGIVKD HSPINLLVGV TMA**HELGHNL**
FIBR_   TAIDFDGDTV GLAYVGGMCQ LKHSTGVIQD HSAINLLVAL TMA**HELGHNL**
ADAM_   TAIEFKCETL GKAYLNSMCN PRSSVGIVKD HSPINLLVAV TMA**HELGHNL**
HR1B_   TTIDFDGPTI GKAYTASMCD PKRSVGIVQD YSPINLVVAV IMT**HEMGHNL**
HR2A_   TDINFTGNTI GWAYMGGMCN AKNSVGIVKD HSSNVFMVAV TMT**HEIGHNL**
HR2_T   TATNLNDNTI GLAYKKGMCN PKLSVGLVQD YSPNVFMVAV TMT**HELGHNL**
HRTD_   TAIELDEETL GLAPLGTMCD PKLSIGIVQD HSPINLLMGV TMA**HELGHNL**
HRTE_   TSIAFDEQII GRAYIGGICD PKRSTGVVQD HSEINLRVAV TMT**HELGHNL**
ACLPF   TAIDFDGDTV GLAYVGGMCQ LKHSTGVIQD HSAINLLVAL TMA**HELGHNL**
ACLPH   TSTDFDGPTI GLAYVGTMCD PKLSTGVVED HSKINFLVAV TMA**HEMGHNL**
                160        170        180        190        200
LEBE_   **GMNHD**G DQC NCGANGCVMS VVLIEQRSYQ FSDCSKNKYQ TYLTNRNPQC ILNQP
ATRO_   **GMNHD**G DKC LRGASLCIMR PGLTPGRSYE FSDDSMGYYQ SFLKQYNPQC IXNK
FIBR_   **GMNHD**G NQC HCGANSCVMA AMLSDQPSKL FSDCSKKDYQ TFLTVNNPQC ILNKP
ADAM_   **GMEHD**G KDC LRGASLCIMR PGLTPGRSYE FSDDSMGYYQ KFLNQYKPQC ILNKP
HR1B_   **GIPHD**G NSC TCGGFPCIMS PMISDPPSEL FSNCSKAYYQ TFLTDHKPQC ILNAP
HR2A_   **GMEHD**DKDKC KCEA CIMS AVISDKPSKL FSDCSKDYYQ TFLTNSKPQC IINAP
HR2_T   **GMEHD**DKDKC KCEA CIMS DVISDKPSKL FSDCSKNDYQ TFLTKYNPQC ILNAP
HRTD_   **GMEHD** GKDC LRGASLCIMR PGLTKGRSYE FSDDSMHYYE RFLKQYKPQC ILNKP
HRTE_   **GIHHD** TDSC SCGGYSCIMS PVISDEPSKY FSDCSYIQCW EFIMNQKPQC ILKKP
ACLPF   **GMNHD**G NQC HCGANSCVMP SVLSDQPSKL FSDCSKKDYQ TFLPVNNPQC ILNKP
ACLPH   **GMRHD**T GSC SCGGYSCIMS PVISDDSPKY FSNCSYIQCW DFIMKENPQC ILNKP
```

FIG. 3. Sequence comparison of mature metalloproteinase (lebetase) deduced from Le3 clone and 10 venom metalloproteinases from various snake species. Sequence alignment was carried out using published sequences of atroxase (9) from *Crotalus atrox*, fibrolase (8) from *Agkistrodon contortrix contortrix*, adamalysin (16) from *Crotalus adamanteus*, HR1B (19), HR2A (20) and HR2 (21) from *Trimeresurus flavoviridis*, HRTD (22) and HRTE (23) from *Crotalus atrox*, ACLPREF and ACLPREH (13) from *Agkistrodon contortrix laticinctus*. The zinc-chelating sequences are in bold.

the medium size proteins. In our case, as in the case of atrolysin e, there is a serine in this position, and the spacer region and disintegrin-like domain observed from the cDNA sequence are processed away. These results support the presence of common precursors for metalloproteinases and disintegrin-like proteins.

The cDNA sequence of lebetase has higher degree of similarity of the 5′ and 3′ untranslated regions and proenzyme regions with other snake venom metalloproteinases when compared with the cDNA regions coding the mature protein. Untranslated sequences of lebetase cDNA have 85-90% identity with *A. c. laticinctus* metalloproteinases untranslated cDNA region. Le3 contains a putative signal peptide (amino acids 1-18.) similar to ACLPREF and ACLPREH (13). Similarities are extensive in the proprotein domain with corresponding domains in ACLPREF and ACLPREH. The variability was higher in the domains present on the mature protein. The amino acid sequence data of fibrinolytic, nonhemorrhagic and hemorrhagic metalloproteinases didn't allow to identify special amino acids which are responsible for fibrinolytic activity. Structural analysis and comparison of various fibrinolytic and hemorrhagic enzymes from disparate snake species show that for discovering the reasons of biological divergency in these enzymes we need the data of crystal structure. Recently these data for nonhemorrhagic metalloproteases adamalysin II from *C. adamanteus* venom (16), H2 from *T. flavoviridis* venom (26) and for hemorrhagic protein atrolysin C from C. atrox venom (27) were published, but there are no structural data about fibrinolytic enzymes.

ACKNOWLEDGMENTS

The work was financially supported by Estonian Science Foundation Grant 1513 and by NIH Grants 5R 37GM 15591 and 2R03 TW 00211. We thank Dr. T. Reintamm for synthesis of oligonucleotide primers. The authors acknowledge the help of S. A. Shepilov in obtaining the venomous gland of the *Vipera lebetina* snake.

REFERENCES

1. Markland, F. S., Jr. (1988) *in* Haemostasis and Animal Venoms (Pirkle, H. and Markland F. S., Eds.), pp. 149–173, Dekker, New York.
2. Siigur, J., and Siigur, E. (1992) *J. Toxicol.-Toxin Rev.* **11,** 93–113.
3. Zhang, Y., Wisner, A., Xiong, Y., and Bon, C. (1995) *J. Biol. Chem.* **270,** 10246–10255.
4. Siigur, J., Samel, M., Tõnismägi, K., and Siigur, E. (1995) *Toxicon* **33,** 258.
5. Siigur, E., Mähar, A., and Siigur, J. (1991) *Toxicon* **29,** 107–118.
6. Siigur, E., and Siigur, J. (1991) *Biochim. Biophys. Acta* **1074,** 223–229.
7. Siigur, J., and Siigur, E. (1993) *Toxicon* **31,** 535.
8. Randolph, A., Chamberlain, S. H., Chu, H.-L., Retzios, A. D., Markland, F. S., and Masiarz, F. R. (1992) *Prot. Sci.* **1,** 590–600.
9. Baker, B. J., Wongvibulsin, S., Nyborg, J., and Tu, A. T. (1995) *Archs. Biochem. Biophys.* **317,** 357–364.
10. Astrup, T., and Müllertz, S. (1952) *Archs. Biochem. Biophys.* **40,** 346–351.
11. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
12. Pearson, W. R., and Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2444–2448.
13. Selistre de Araujo, H. S., and Ownby, C. L. (1995) *Arch. Biochem. Biophys.* **320,** 141–148.
14. Bjarnason, B., and Fox, J. W. (1995) *Methods Enzymol.* **248,** 345–368.
15. Gomis-Rüth, F-X., Kress, L. F., and Bode, W. (1993) *EMBO Journal* **12,** 4151–4157.
16. Gomis-Rüth, F-X., Kress, L. F., Kellermann, J., Mayr, I., Lee, X., Huber, R., and Bode, W. (1994) *J. Mol. Biol.* **239,** 513–544.
17. Fox, J. W., and Bjarnason, B. (1995) *Methods Enzymol.* **248,** 368–387.
18. Manning, M. C. (1995) *Toxicon* **33,** 1189–1200.
19. Takeya, H., Oda, K., Miyata, T., Omori-Satoh, T., and Iwanaga, S. (1990) *J. Biol. Chem.* **265,** 16068–16073.
20. Miyata, T., Takeya, H., Ozeki, Y., Arakawa, M., Tokunaga, F., Iwanaga, S., and Omori-Satoh, T. (1989) *J. Biochem. Tokyo* **105,** 847–853.
21. Takeya, H., Arakawa, M., Iwanaga, S., and Omori-Satoh, T. (1989) *J. Biochem. Tokyo* **106,** 151–157.
22. Hite, L. A., Jia, L.-G., Bjarnason, J. B., and Fox, J. W. (1994) *Archs. Biochem. Biophys.* **308,** 182–191.
23. Hite, L. A., Shannon, J. D., Bjarnason, J. B., and Fox, J. W. (1992) *Biochemistry* **31,** 6203–6211.
24. Hooper, N. M. (1994) *FEBS Lett.* **354,** 1–6.
25. Grams, F., Huber, R., Kress, L. F., Moroder, L., and Bode, W. (1993) *FEBS Lett.* **335,** 76–78.

26. Kumasaka, T., Yamamoto, M., Moriyama, H., Tanaka, N., Sato, M., Katsube, Y., Yamakawa, Y., Omori-Satoh, T., Iwanaga, S., and Ueki, T. (1996) *J. Biochem. Tokyo* **119,** 49–57.
27. Zhang, D., Botos, I., Gomis-Rüth, F-X., Doll, R., Blood, C., Njoroge, F. G., Fox, J. W., Bode, W., and Meyer, E. F. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8447–8451.