ELSEVIER

Journal of Chromatography A, 852 (1999) 237–243

JOURNAL OF CHROMATOGRAPHY A

Partial primary structure of a fibrinogenase from the venom of the snake *Lachesis stenophrys*

Adrijana Leonardi[a,*], Federico Aragon-Ortiz[b], Franc Gubenšek[a], Igor Križaj[a]

[a]*Department of Biochemistry and Molecular Biology, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia*
[b]*Department of Biochemistry, School of Medicine, University of Costa Rica, San Jose, Costa Rica*

Abstract

The partial primary structure of an M_r 24 000 non-haemorrhagic metalloproteinase isolated from the venom of the snake *Lachesis stenophrys* has been determined. The native proteinase was resistant to Edman degradation exhibiting the *N*-terminal blockade. The pyridylethylated or native proteinase was chemically and enzymatically fragmented and the obtained peptides were separated by gel or reversed-phase chromatography, and sequenced. The metalloproteinase from *Lachesis stenophrys* contains a putative zinc-chelating sequence HELGHNLGMKH, characteristic for the reprolysin family of zinc-metalloproteinases. It contains six cysteine residues in the standard positions for this group of proteins suggesting the same disulfide bonding. Interestingly, it has almost identical sequence as the metalloproteinase from *Lachesis muta muta*, LHF-II, which is, however, haemorrhagic. The main structural differences between the two molecules were found in their *N*-terminal parts and in glycosylation. As the substrate-binding regions of both proteinases are practically identical, we suggest that the absence of haemorrhagicity in *Lachesis stenophrys* enzyme is due to its lower affinity for the matrix proteins and not due to different substrate specificity.

Keywords: *Lachesis stenophrys*; Venoms; Fibrinogenases; Enzymes; Metalloproteinases

1. Introduction

Snake venom contains a large number of zinc metalloproteinases, which exhibit various pathological effects on blood coagulation, fibrinolysis and complement system [1]. Despite differences in their molecular masses and activities, they all appear to be related through a single ancestral gene, as observed by the comparison of their primary structures [2]. They are synthesized in the venom gland as zymogens and subsequently processed to the active forms. These proteinases are divided into four classes, P-I to P-IV, according to their domain structure [3]. The proteinase domains in all classes are structurally homologous. One of the most characteristic structural elements is the extended zinc-binding sequence HExxHxxGxxH. The three-dimensional structures of three zinc-metalloproteinases, adamalysin II [4], atrolysin C [5], and H_2-proteinase [6] revealed another conserved, five amino acid long methionine-containing structural motif, the Met-turn. The high-molecular-mass proteinases have, in addition to proteinase domain, 1–3 non-enzymatic domains located C-terminally to the proteinase domain.

To date, many snake venom metalloproteinases have been characterized and their amino acid sequences determined in order to elucidate the structural basis of their pathopharmacological actions [1,7–17]. To provide further information about the structural elements which determine the haemorrhagicity of some of them we have attempted to solve the

*Corresponding author.
E-mail address: Adrijana.Colic@IJS.SI (A. Leonardi)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00260-5

amino acid sequence of an M_r 24 000 fibrinogenase from snake *Lachesis stenophrys* (LSF). LSF is one of the major metalloproteinases found in *L. stenophrys* venom. The proteinase cleaves Aα and Bβ chains of fibrinogen, and has no haemorrhagic activity [18]. It is glycosylated and belongs to the P-I class of snake venom metalloproteinases.

2. Experimental

2.1. Materials

LSF was purified from *L. stenophrys* venom, as described [18]. Sephacryl S-200 HR was from Pharmacia Biotech (Uppsala, Sweden), formic acid from Merck (Darmstadt, Germany) and acetonitrile from Rathburn (Walkerburn, UK). CNBr was purchased from Pierce (Rockford, USA), 4-vinylpyridine and trypsin (EC 3.4.21.4.) were from Sigma (St. Louis, MO, USA). *Staphylococcus aureus*, strain V8, proteinase (EC 3.4.21.19) was from Miles (Naperville, UK). Sequencing reagents were obtained from PE Applied Biosystems (Foster City, CA, USA). All other chemicals used were of analytical or sequential grade.

2.2. S-Pyridylethylation

The proteinase was pyridylethylated according to the procedure of Henschen [19]. Following the reaction, protein was desalted on Sephacryl S-200 HR column as described in Section 2.6. Fractions of 0.6 ml were collected and pooled according to absorbance at 280 nm.

2.3. CNBr cleavage of pyridylethylated metalloproteinase and CN2 peptide

One hundred and seventy nmol of pyridylethylated proteinase was dissolved in 300 μl of 80% (v/v) aqueous formic acid and 5 μl of β-mercaptoethanol was added. After the addition of CNBr in 50-fold molar excess over the proteinase, the reaction proceeded in the dark for 24 h. The cleavage was terminated by 5-fold dilution of the reaction mixture with water and freeze drying. The reaction products were not completely soluble in water. The water-soluble peptides were separated on the reversed-phase high-performance liquid chromatography (HPLC) as described in Section 2.6. Peptides were analyzed for their amino acid composition before sequencing. The water-insoluble peptides were dissolved in 50% (v/v) aqueous formic acid and separated by Sephacryl S-200 HR chromatography (Section 2.6).

More rigorous CNBr cleavage conditions were used to subcleave CN2 peptide further at tryptophan residues. CN2 peptide was dissolved in 200 μl of 80% (v/v) aqueous formic acid and 100-fold molar excess of CNBr over CN2 peptide was added. The reaction time was extended from 24 to 48 h. The procedures which followed were the same as described above.

2.4. Tryptic digestion

The native proteinase (1.7 nmol) was incubated with trypsin for 70 min at 37°C in *N*-methylmorpholine acetate buffer, pH 8.1. After 30 min of incubation new portion of trypsin was added to a final LSF–trypsin ratio of 50:1 (w/w). The sample was analysed by reversed-phase HPLC as described in Section 2.6. A linear gradient of acetonitrile to 60% (v/v) in 30 min, was used.

2.5. S. aureus V8 proteinase digestion

The native proteinase was treated with *S. aureus* V8 proteinase in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. SDS–PAGE was carried out in 15% gel using Hoeffer Mighty Small II apparatus (Pharmacia Biotech, San Francisco, CA, USA) according to Laemmli [20]. One μg of *S. aureus* V8 proteinase was loaded in the well and allowed to enter the stacking gel. Subsequently, 1 nmol of native metalloproteinase, dissolved in non-reducing SDS–PAGE buffer, was loaded in the same well and electrophoresis started. After proteins reached the stacking–separating gel interphase, the current was switched off and the proteolysis was allowed to take place. The reaction was terminated after 60 min by restarting the electrophoresis. At the same time, refer-

ence lines containing only *S. aureus* V8 proteinase or native LSF were run. Resulting peptides were electroblotted onto Sequi-Blot PVDF membrane (Bio-Rad, Hercules, CA, USA) according to Ref. [21] using a semi-dry transfer unit from Biometra (Göttingen, Germany). Peptides were visualized by Coomassie Brilliant Blue R250 staining, excised and sequenced.

2.6. Chromatographic methods

Gel filtration chromatography was performed on a Sephacryl S-200 HR column (150×0.5 cm) with 50% (v/v) aqueous formic acid as a mobile phase. The flow-rate was 2.4 ml/h and absorbance at 280 nm was continuously monitored. The column was calibrated using bovine serum albumin (M_r 67 000), cytochrome *c* (M_r 12 300), insulin B-chain (M_r 3500) and tryptophan (M_r 200) as molecular mass standards.

The reversed-phase HPLC was performed on HP1100 liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) using a Vydac 218TP52 Protein/Peptide reversed-phase column, 25 cm×0.46 mm, 5 μm particle size (Separations Group, Hesperia, CA, USA). The sample was injected on the column equilibrated with 0.1% (v/v) trifluoroacetic acid in water. A linear gradient of acetonitrile to 40% (v/v) in 30 min was used to elute the bound peptides. The flow-rate was 0.5 ml/min. Peptides were detected by absorbance at 215 nm and collected manually.

2.7. Peptide sequencing

Automated sequence analyses were performed on Procise protein sequencing system 492A (PE Applied Biosystems).

2.8. Amino acid analysis

Samples were hydrolyzed in 6.0 *M* HCl at 110°C for 24 h. Analysis of the hydrolysates were performed by 421A amino acid analyzer (PE Applied Biosystems).

3. Results and discussion

3.1. Sequence determination

We have determined almost complete amino acid sequence of a non-haemorrhagic metalloproteinase from *L. stenophrys* venom, LSF. The native LSF was resistant to Edman degradation, indicating that the amino terminus of the protein is blocked, as reported for some other snake venom proteinases [1,4,9,17]. The basic set of peptides was generated by CNBr cleavage of pyridylethylated LSF (peptides CN2–CN8 in Fig. 1). CNBr peptides were properly ordered after sequencing the overlapping peptides obtained from the native molecule by digestion with either trypsin or *S. aureus* V8 proteinase (Fig. 2). Peptides CN5 and CN7 resulted from incomplete hydrolysis and facilitated the alignment of the CN peptides. CN2 peptide, which corresponds to almost half of the entire molecule, was sequenced up to its 53rd amino acid residue. To complete the sequence, extended CNBr cleavage was made to subcleave the peptide CN2 at tryptophan residues (CN2W peptides in Fig. 2). The sequence of CN2W3 became unclear after the 23rd cycle. The native LSF was submitted to *S. aureus* V8 proteinase digestion and peptide V1 resulted from an unspecific cleavage after Leu95. It provided the overlap between peptides CN2 and CN3. Another set of peptides was prepared from the native LSF with trypsin (T peptides in Fig. 2). Tryptic peptides provided information on the amino-terminus of the molecule and filled the gap between residues 130–139.

As seen from Fig. 2, LHF-II has a potential *N*-linked glycosylation site at Asn70. During sequence analysis of peptides CN2 and CN2W2 from LSF, Asn70 was unambiguously determined suggesting that it does not contain linked sugars. However, amino acid at position 72, which is Thr in LHF-II, gave a blank cycle during sequencing of LSF raising possibility that it has an *O*-linked sugar moiety.

3.2. Sequence comparison

When LSF partial amino acid sequence was aligned with the sequences of other snake venom metalloproteinases belonging to the reprolysin family

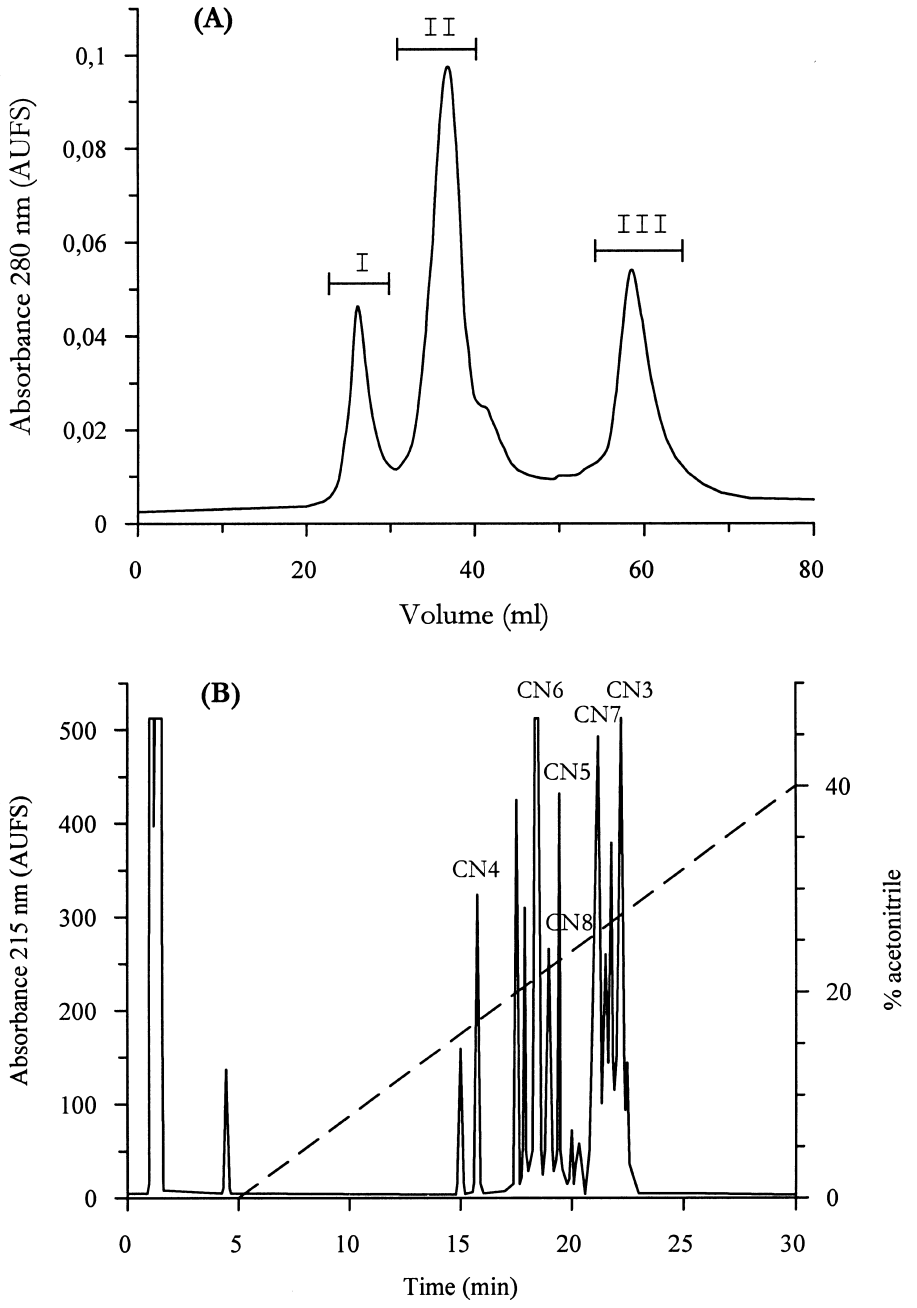

Fig. 1. The separation of peptides obtained after CNBr cleavage of pyridylethylated LSF. (A) Gel filtration chromatography of water-insoluble peptides on a Sephacryl S-200 HR column (150×0.5 cm) equilibrated with 50% (v/v) aqueous formic acid. The flow-rate was 2.4 ml/min. Fractions of 0.6 ml were collected, pooled as indicated and analyzed. The uncleaved LSF was detected in pool I, while in pool II CN2 peptide was found. Salts eluted from the column in peak III. (B) HPLC of water-soluble peptides on a Vydac 218TP52 Protein/Peptide reversed-phase column (25 cm×0.46 mm, 5 μm particle size) equilibrated in 0.1% (v/v) trifluoroacetic acid in water. The peptides were eluted by a linear gradient of acetonitrile. A flow-rate of 0.5 ml/min was used. The elution positions of the peptides used in the determination of LSF sequence (Fig. 2) are indicated.

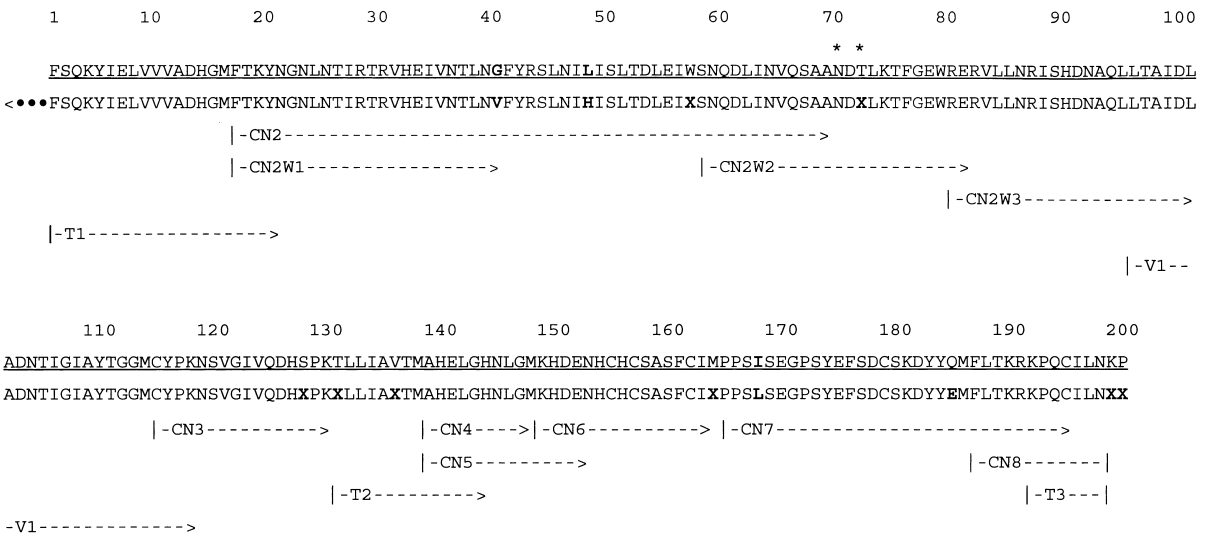

Fig. 2. The partial amino acid sequence of a non-haemorrhagic metalloproteinase from *L. stenophrys*, strategy of its determination and its comparison with the sequence of a haemorrhagic metalloproteinase, LHF-II, from *L. muta muta* venom (underlined) [7]. Abbreviations: CN and CN2W for peptides obtained after CNBr cleavage of pyridylethylated LSF and its CN2 peptide, respectively; T for peptides from tryptic and V for the peptide from *S. aureus* V-8 proteinase digestions of the native LSF. <●●● Depicts blocked amino terminal extension. Unidentified amino acid residues are indicated by X. The amino acids that are different in both sequences are bold. Potential *N*- and *O*-glycosylated amino acid residues are indicated by asterisks.

of zinc-metalloproteinases (Fig. 3) two characteristic structural motifs were clearly recognised in LSF: 1. the zinc-binding consensus sequence HELGHNLGMKH and 2. the Met-turn with the conserved proline in position five in the consensus sequence CIMPP.

Snake venom metalloproteinases contain from four to eight cysteine residues that form two to four disulfide bonds, one being conserved among all of them. Based on the sequence homology with other snake venom metalloproteinases, six cysteines were found in the standard positions at the *C*-terminus of LSF. These tentatively form three disulfide bonds as follows Cys115–Cys196, Cys156–Cys180 and Cys158–Cys163 (Fig. 3).

Up to now, no clear structural differences have been found between haemorrhagic and non-haemorrhagic metalloproteinases. A comparison of the LSF partial primary structure with structures of other snake venom metalloproteinases reveals that it is almost identical to the sequence of metalloproteinase from *L. muta muta*, LHF-II, which is haemorrhagic [7]. A haemorrhage is thought to be due to disruption of the extracellular matrix proteins in the basement membrane of the vessel wall, which forms a barrier against erythrocyte escape [23]. The substrate-binding region, which is located in the *C*-terminal part of the molecule, is almost identical in both proteinases, suggesting that their substantially different patopharmacological activities do not stem from their different substrate specificities. Instead, they might differ in their affinities for the matrix proteins as a result of subtle structural differences in the *N*-terminal parts. These differences comprise elongated and blocked *N*-terminus in *L. stenophrys* enzyme, three amino acid substitutions at positions 40, Val→Gly, 48, His→Leu, and 185, Gln→Glu (Fig. 2), and a difference in glycosylation. LSF is a glycoprotein [18], while LHF-II contains a potential glycosylation site but is not glycosylated [7]. A carbohydrate moiety could sterically hinder the binding of LSF to the matrix proteins, thus lowering its specific enzyme activity and consequently its haemorrhagicity.

4. Conclusions

We report the partial amino acid sequence of a

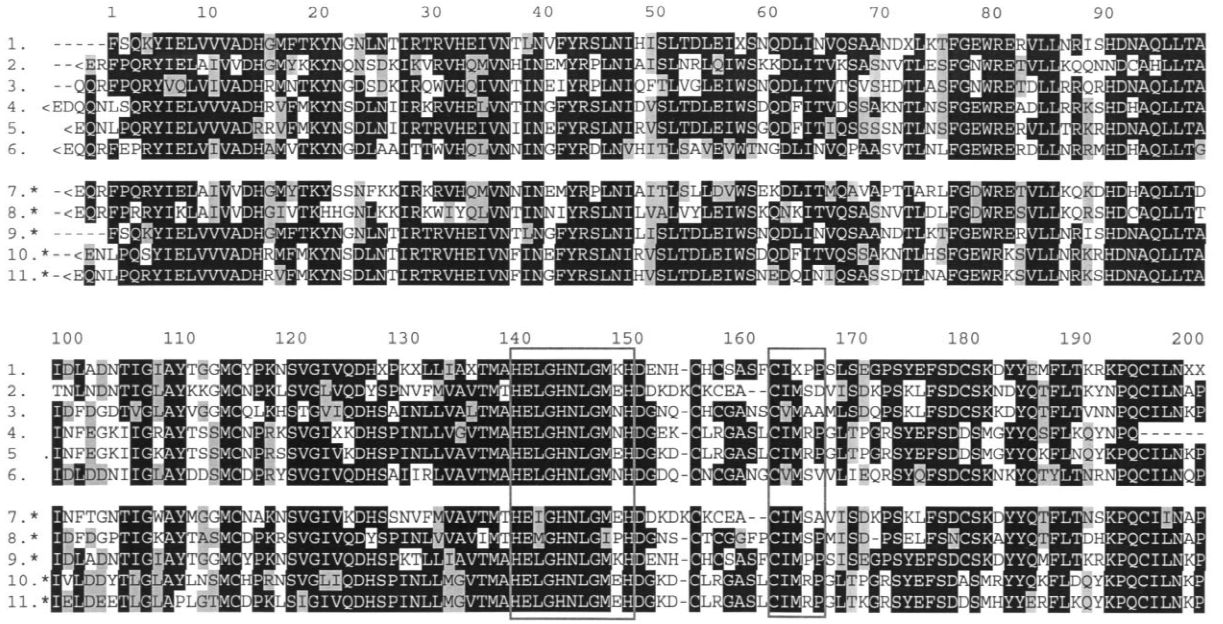

Fig. 3. Alignment of the amino acid sequences of non-haemorrhagic and haemorrhagic (*) snake venom metalloproteinases. 1=LSF, fibrinogenolytic metalloproteinase from *L. stenophrys* (this work); 2=H_2, metalloproteinase from *Trimeresurus flavoviridis* [1]; 3=fibrolase, fibrinolytic proteinase from *Agkistrodon contortrix contortrix* [9]; 4=atroxase, the partial sequence of fibrinolytic proteinase from *Crotalus atrox* [12]; 5=adamalysin II, Zn endopeptidase from *C. adamanteus* [4]; 6=lebetase, fibrinolytic metalloproteinase from *Vipera lebetina* [17]; 7=HR2a, metalloproteinase from *T. flavoviridis* [1]; 8=HR1B, metalloproteinase domain of the high-molecular-mass metalloproteinase from *T. flavoviridis* [1]; 9=LHF-II, haemorrhagic factor from *L. muta muta* [7], 10=HT-2, metalloproteinase from *C. ruber ruber* [1]; 11=Ht-d, metalloproteinase from *C. atrox* [1]. Zn-binding consensus sequence (residues 140–150) and Met-turn (residues 163–167) are boxed. Identical and similar residues are highlighted. <E, pyroglutamic acid. Numbering refers to LHF-II. Alignment was made using the program CLUSTAL W [22].

non-haemorrhagic snake metalloproteinase, LSF, from *L. stenophrys* venom. Structural characteristics, e.g., zinc-binding site and Met-turn place LSF in the reprolysin family of zinc-metalloproteinases. The high level of sequence identity between non-haemorrhagic LSF and haemorrhagic metalloproteinase LHF-II from *L. muta muta* venom, suggests that they differ in their affinities for binding matrix proteins as a consequence of structural differences at their *N*-terminal parts and/or glycosilation.

References

[1] R.M. Kini, H.J. Evans, Toxicon 30 (1992) 265.
[2] L.A. Hite, J.D. Shanon, J.B. Bjarnason, J.W. Fox, Biochemistry 31 (1992) 6203.
[3] L.G. Jia, K.I. Shimokava, J.B. Bjarnason, J.W. Fox, Toxicon 34 (1996) 1269.
[4] F.X. Gomis-Rüth, L.F. Kress, W. Bode, EMBO J. 11 (1993) 4151.
[5] D. Zhang, I. Botos, F.X. Gomis-Rüth, R. Doll, C. Blood, F.G. Njoroge, J.W. Fox, W. Bode, E.F. Meyer, Proc. Natl. Acad. Sci. USA 91 (1994) 8447.
[6] T. Kumasaka, M. Yamamoto, H. Moriyama, N. Tanaka, M. Sato, Y. Katsube, Y. Yamakawa, T. Omori-Satoh, S. Iwanaga, T. Ueki, J. Biochem. 119 (1996) 49.
[7] E.F. Sanchez, C.R. Diniz, M. Richardson, FEBS Lett. 282 (1991) 178.
[8] M.J.I. Paine, H.P. Desmond, R.D.G. Theakston, J.M. Crampton, J. Biol. Chem. 267 (1992) 22869.
[9] A. Randolph, S.H. Chamberlain, H.L. Chu, A.D. Retzios, F.S. Markland Jr., F.R. Masiarz, Protein Sci. 1 (1992) 590.
[10] L.C. Au, J.C. Chou, K.J. Chang, G.W. Teh, S.B. Lin, Biochim. Biophys. Acta 1173 (1993) 243.
[11] L.A. Hite, L.G. Jia, J.B. Bjarnason, J.W. Fox, Arch. Biochem. Biophys. 308 (1994) 182.

[12] M.J. Paine, A.M. Moura-Da-Silva, R.D.G. Theakston, J.M. Crampton, Eur. J. Biochem. 224 (1994) 483.
[13] M. Iha, Z.Q. Qi, T. Kannki, Y. Tomihara, K. Yonaha, Toxicon 33 (1995) 229.
[14] A.T. Tu, B. Baker, S. Wongvibulsin, T. Willis, Toxicon 34 (1996) 1295.
[15] M.C.M. Chung, G. Ponnudurai, M. Kataoka, S. Shimizu, N.H. Tan, Arch. Biochem. Biophys. 325 (1996) 199.
[16] K. Shimokawa, L.G. Jia, X.M. Wang, J.W. Fox, Arch. Biochem. Biophys. 335 (1996) 283.
[17] E. Siigur, A. Aaspollu, A.T. Tu, J. Siigur, Biochem. Biophys. Res. Commun. 224 (1996) 229.
[18] F. Aragon-Ortiz, Rev. Biol. Trop. 40 (1992) 209.
[19] A. Henschen, in: B. Wittmann-Liebold, J. Salnikov, V.A. Erdmann (Eds.), Advanced Methods in Protein Microsequence Analysis, Springer, Berlin, 1986, p. 244.
[20] U.K. Laemmli, Nature 227 (1970) 680.
[21] S. Gallagher, S.E. Winston, S.A. Fuller, J.G.R. Hurell, in: F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds.), Current Protocols in Molecular Biology, Vol. 2, John Wiley and Sons, Inc, USA, 1997, 10.8.4.
[22] J.D. Thompson, D.G. Higgins, T.J. Gibson, Nucleic Acids Res. 22 (1994) 4673.
[23] A.S. Kamiguti, C.R.M. Hay, R.D.G. Theakston, M. Zuzel, Toxicon 34 (1996) 627.