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The complete amino acid sequence of the haemorrhagic factor LHFII, a metalloproteinase isolated from the venom of the bushmaster snake (*Lachesis muta muta*)

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The complete amino acid sequence the haemorrhagic agent LHFII, a Zn and Cu containing metalloproteinase isolated from the venom of the Bushmaster snake *Lachesis muta muta* was determined by automated and DABITC/PITC microsequencing of the intact protein, fragments derived by cleavage with cyanogen bromide, and peptides resulting from enzymatic digestions with trypsin and the protease from *S. aureus* V8. The protein is composed of 200 residues and exhibits considerable sequence homology with the haemorrhagic toxins from a number of other snake venoms, and some metalloproteinases in the region of the putative Zn-binding sites.

Snake venom; Amino acid sequence; Metalloproteinase; Hemorrhagic factor; *Lachesis muta muta*

1. INTRODUCTION

The bushmaster (*Lachesis muta muta*), a pit viper, occurs in forested regions of equatorial South America. The venom of this large crotalid snake is notable for its haemorrhagic, proteolytic and blood clotting activities [1–4]. The haemorrhagic activity in the venom has been ascribed to the presence of metalloproteinases [5–8] which are thought likely to damage the pericellular basement membrane and subsequently attack the walls of the blood vessels. Workers at the Fundacão Ezequiel Dias in Brazil have recently purified two such haemorrhagic factors from the venom of *L. muta muta*, LHF-I, a high molecular weight factor with high substrate specificity [2], and LHF-II, a low molecular weight factor [9].

LHF-II was obtained in an electrophoretically and immunologically homogeneous form with a pI of 6.6 [9]. It was shown to have a molecular weight of 22300 and to contain 1 g atom Zn and 2 g atoms Ca per mole of protein. The factor had proteolytic activity towards several substrates including fibrinogen, in which the A chain was hydrolyzed selectively. The metalloproteinase nature of the factor was indicated by its activation with Ca^{2+} and inhibition by Zn^{2+}, cysteine and metal chelators such as EDTA, EGTA and 1:10-phenanthroline.

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We report here the complete primary structure of the *L. muta muta* haemorrhagic factor LHF-II and its sequence homology with some other metalloproteinases recently reported from snake venoms.

2. MATERIALS AND METHODS

Venom of *L. muta muta* was obtained by milking specimens captured near Manaus (Amazonas, Brazil) and maintained in the Serpentarium of the Fundacão Ezequiel Dias, Belo Horizonte, Brazil.

The haemorrhagic factor LHF-II was purified from the crude venom by gel filtration on Sephadex G-100 and G-50, and by ion exchange chromatography on CM-Sepharose as described in [9]. A sample (5 nmol) of the protein was reduced and S-alkylated in situ on a sequencer filter disc using 4-vinylpyridine and tributylphosphine in the vapour phase as described in [10]. This sample was subjected to automatic sequencing in an Applied Biosystems Model 477A pulsed liquid phase Sequencer/Model 120A PTH HPLC analyzer system using a standard Edman degradation programme.

A further sample (1 mg) was reduced and *S*-carboxymethylated as described in [11], and treated with cyanogen bromide (100-fold molar excess) in 70% formic acid for 24 h at 20°C. The fragments which resulted were lyophilized and purified by reverse phase HPLC in a Varian 5000 HPLC fitted with a Vydac C18 column (25 cm × 4.6 mm; 218TP54, Technicol, Stockport) using a linear gradient of 0–70% acetonitrile (HPLC grade S, Rathburn) in 0.1% aqueous trifluoroacetic acid. Other samples (1 mg) of the reduced and *S*-carboxymethylated protein were digested separately with trypsin and the Glu-specific protease from *S. aureus* V8 as described in [12]. The peptides produced were purified by reverse phase HPLC as described above. The larger peptides from the CNBr cleavage and enzyme digests were sequenced by automated means using 2–7 nmol amounts. The smaller fragments (10–20 nmol) were subjected to a manual method, 4-*N,N*-dimethylaminoazobenzene-4′-isothiocyanate (DABITC)/phenylisothiocyanate (PITC) double coupling microsequencing [13].

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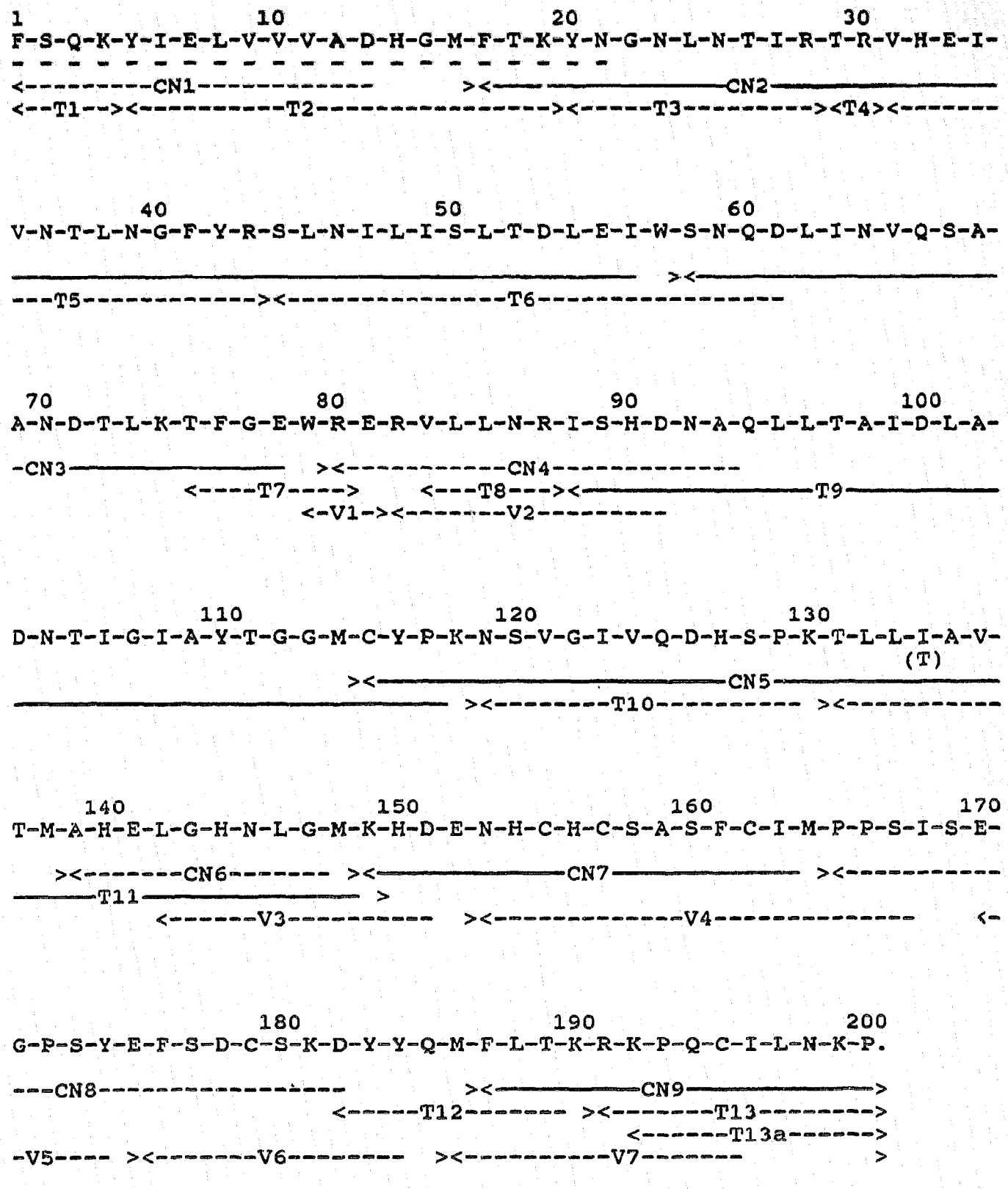

Fig. 1. The amino acid sequence of the haemorrhagic metalloproteinase LHF-II from the venom of the bushmaster snake *Lachesis muta muta*. CN, fragments from cleavage with cyanogen bromide; T, peptides from digestion with trypsin; V, peptides from digestion with *S. aureus* V8 protease; →, residues sequenced from N-terminal of intact protein by automated degradation in pulsed liquid phase sequencer. <⟶> fragments sequenced by automated means; <---->, fragments sequenced by DABITC/PITC manual method.

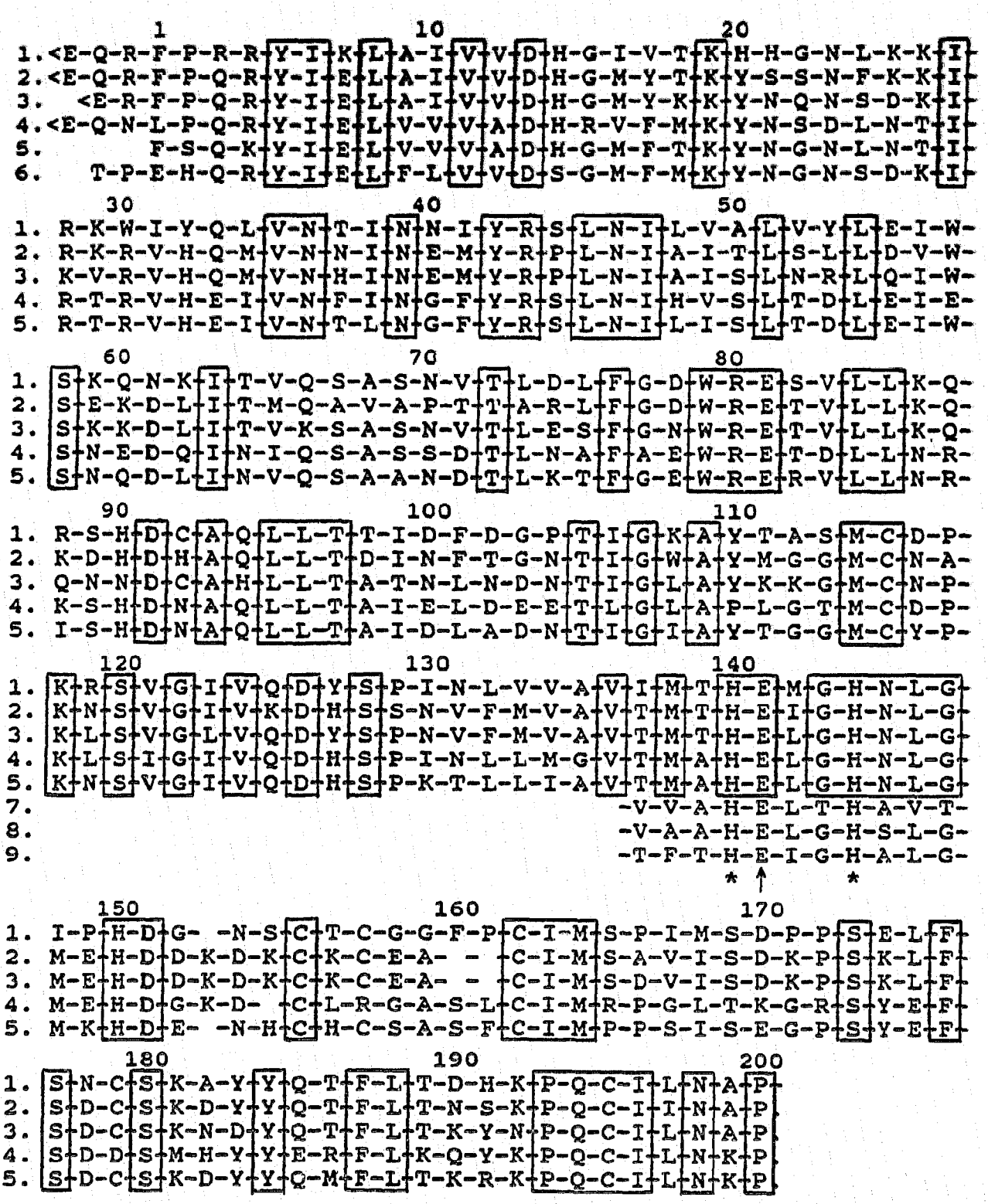

Fig. 2. Alignments of the amino acid sequences of haemorrhagic and non-haemorrhagic metalloproteinases from snake venoms and the putative Zn-binding regions of some other metalloproteinases. 1, N-terminal region (metalloproteinase domain) of the high molecular weight haemorrhagic HR1B from *Trimeresurus flavoviridis* [17]; 2, low molecular weight haemorrhagic protein HR2a from *T. flavoviridis* [18]; 3, non-haemorrhagic metalloproteinase H_2 from *T. flavoviridis* [19]; 4, haemorrhagic metalloproteinase toxin d from *Crotalus atrox* [20]; 5, haemorrhagic factor LHF-II from *Lachesis muta muta* (this work); 6, N-terminal fragment of Zn metalloproteinase from venom of *Bothrops jararaca* [21]; putative Zn-binding regions of 7, thermolysin [22]; 8, human collagenase [23]; 9, Zn protease from *Serratia* [24]. Numbering refers to residues of *Lachesis* LHF-II. Boxes enclose residues which are the same in all of the snake proteins. The asterisks mark the putative Zn ligands, and arrows the catalytic sites. <E, pyroglutamic acid.

Samples (50 μg) for amino acid analysis were hydrolyzed with 5.6 M HCl containing 0.02% (v/v) cresol at 108°C for 24 h. The hydrolyzates were derivatized with phenylisothiocyanate and analyzed by HPLC using the Waters Pico-Tag method [14].

The amino acid sequence of LHF-II was compared with those of other proteins stored in the US National Biomedical Research Foundation Databank (1990) by computer analysis using the FASTA programme [15].

3. RESULTS AND DISCUSSION

The amino acid sequence of the *Lachesis muta muta* LHF-II haemorrhagic factor determined by both automated and manual sequencing methods is shown in Fig. 1. The determination of the sequence was facilitated by the isolation of two fragments (CN3 and CN4) in relatively low yield, which resulted from the non-specific cleavages by cyanogen bromide of the Trp^{57}-Ser^{58} and Trp^{79}-Arg^{80} peptide bonds. This type of cleavage of Trp-X peptide bonds by cyanogen bromide is not uncommon when high concentrations of cyanogen bromide are employed [12,16]. The amino acid sequence is in good agreement with the results of amino acid analyses, and the M_r of 22581 calculated from the sequence corresponds well with the estimates of 22300 and 21600 made from SDS-PAGE and ultracentrifuge sedimentation methods, respectively [9]. Only one example of sequence microheterogeneity was detected in the protein and that occurred in position 134 where minor amounts (15%) of Thr were found in addition to the Ile.

The structure of LHF-II shows clear and strong sequence similarities with the recently reported haemorrhagic and non-haemorrhagic metalloproteinases from the venoms of the snakes *Trimeresurus flavoviridis* [17–19], *Crotalus atrox* [20] and *Bothrops jararaca* [21]. Inspection of the alignments of these proteins shown in Fig. 2 reveals that some 70 out of 200–203 residues (approx. 35%) are conserved in all of the proteins, moreover many of the alterations of amino acids involve only substitutions with very similar amino acids. It is noteworthy, however, that of the 7 positions in which Cys residues have been found only four are conserved in all of the proteins. The sequence of the *Lachesis* LHF-II shows most homology (68.5% of identical residues) with the haemorrhagic metalloproteinase toxin d from the venom of the other crotalid *Crotalus atrox* [20], and approximately 60% similarity with the various proteins from *Trimeresurus flavoviridis*.

The putative metalloproteinase zinc-chelating sequence of LHF-II is clearly recognisable as residues 137–148 by virtue of the zinc-chelating His^{140}, His^{144} and catalytic Glu^{141} residues identified by homology to the zinc-binding regions (His^{142}, His^{146} and Glu^{143}) of thermolysin [22], human collagenase [23] and the *Serratia* Zn protease [24]. Furthermore when the secondary structure of LHF-II was predicted from the amino acid sequence by the method of Garnier et al. [25] it was found that the putative Zn-chelating region was situated in the middle of a stretch (residues 132–154) of α-helix in the same manner as in the other snake venom metalloproteinases and thermolysin. It is interesting to note that the proteolytic specificity of the *Lachesis* LHF-II closely resembles thermolysin in that it only hydrolyzed the peptide bonds on the N-terminal side of Leu residues when the oxidized B chain of insulin was employed as a substrate (Sanchez and Cordeiro, unpublished results).

The sequence of LHF-II also contains a potential N-linked glycosylation sequence in the residues Asn^{70}-Asp^{71}-Thr^{72} and we are currently investigating the possible attachment of carbohydrate in this region. The yield of the Pth-Asn obtained in position 70 during the automated degradation of peptide CN3 and the absence of other forms of this peptide suggests that LHF-II probably has no N-linked carbohydrate. Although both the haemorrhagic HR1B and the non-haemorrhagic H_2 proteinase from *T. flavoviridis* contain a similar glycosylation sequence in the homologous position, only the former had sugar chains linked to it [17,19].

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REFERENCES

[1] Deutsch, H.F. and Diniz, C.R. (1955) J. Biol. Chem. 216, 17–26.
[2] Sanchez, E.F., Magalhaes, A. and Diniz, C.R. (1987) Toxicon 25, 611–619.
[3] Da Silva, N.J., Aird, S.D., Seebart, C. and Kaiser, I.I. (1989) Toxicon 27, 763–771.
[4] Silveira, A.M.V., Magalhaes, A., Diniz, C.R. and De Oliveira, E.B. (1989) Int. J. Biochem. 21, 863–871.
[5] Bjarnason, J.B. and Tu, A.T. (1978) Biochemistry 17, 3395–3404.
[6] Nikai, T., Ishisaki, H., Tu, A.T. and Sugihara, H. (1982) Comp. Biochem. Physiol. 72C, 103–106.
[7] Mandelbaum, F.R., Assakura, M.T. and Reichl, A.P. (1984) Toxicon 22, 193–206.
[8] Bjarnason, J.B. and Fox, J.W. (1989) J. Toxic. Toxin Rev. 7, 121–209.
[9] Sanchez, E.F., Magalhaes, A., Mandelbaum, F.R. and Diniz, C.R. (1991) Biochim. Biophys. Acta (in press).
[10] Amons, R. (1987) FEBS Lett. 212, 68–72.
[11] Crestfield, A.M., Moore, S. and Stein, W.H. (1963) J. Biol. Chem. 238, 622–627.
[12] Aitken, A., Geisow, M.J., Findlay, J.B.C., Holmes, C. and Yarwood, A. (1989) in: Protein Sequencing: A Practical Approach (Findlay, J.B.C. and Geisow, M.J. eds) p. 43, IRL Press, Oxford.
[13] Chang, J.Y., Brauer, D. and Wittman-Liebold, B. (1978) FEBS Lett. 93, 205–214.

[14] Millipore Waters Chromatography Corp. (1984) Operaters Manual No. 88140.
[15] Pearson, W.R. and Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2444–2448.
[16] Oxals, J. and Gerhard, C. (1977) J. Biol. Chem. 252, 5986–5990.
[17] Takeya, H., Oda, K., Miyata, T., Omori-Satoh, T. and Iwanaga, S. (1990) J. Biol. Chem. 265, 16068–16073.
[18] Miyata, T., Takeya, H., Ozeki, Y., Arakawa, M., Tokunaga, F., Iwanaga, S. and Omori-Satoh, T. (1989) J. Biochem. 105, 847–853.
[19] Takeya, H., Arakawa, M., Miyata, T., Iwanaga, S. and Omori-Satoh, T. (1989) J. Biochem. 106, 151–157.
[20] Shannon, J.D., Baramova, E.N., Bjarnason, J.B. and Fox, J.W. (1989) J. Biol. Chem. 264, 11575–11583.
[21] Tanizaki, M.M., Zingali, R.B., Kawasaki, H., Imajoh, S., Yamazaki, S. and Suzuki, K. (1989) Toxicon 27, 747–755.
[22] Matthews, B.W., Jansonius, J.M., Colman, P.M., Scoenborn, B.P. and Dupourque, D. (1972) Nature New Biol. 238, 37–41.
[23] Goldberg, G.I., Wilhelm, S.M., Kronberger, A., Bauer, E.A., Grant, G.A. and Eisen, A.Z. (1986) J. Biol. Chem. 261, 6600–6605.
[24] Nakahama, K., Yoshimura, K., Marumoto, R., Kikuchi, M., Lee, I.S., Hase, T. and Matsubara, H. (1986) Nucleic Acids Res. 14, 5843–5855.
[25] Garnier, J., Osguthorpe, J.D. and Robson, B. (1978) J. Mol. Biol. 120, 97–120.