AMINO ACID SEQUENCE OF CARDIOTOXIN FROM THE VENOM OF *NAJA NAJA ATRA*

Kyozo HAYASHI*, Masayuki TAKECHI, Norio KANEDA and Toyosaku SASAKI**
Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

Received 17 April 1976

1. Introduction

In addition to neurotoxin, certain *Elapidae* venoms, i.e. those from the genera *Naja* and *Haemachatus*, contain cardiotoxin homologues (cytotoxins) as their major protein constituents [1]. Recently, a number of publications have appeared on the amino acid sequences of snake venom cardiotoxins [2–15].

During the course of our study on the purification and characterization of toxic components of the venom of *Naja naja atra*, we separated four cardiotoxic proteins. In our previous report [5], we described the sequence of cardiotoxin analogue I, one of the above four cardiotoxins. It has been shown that these proteins are highly homologous to the neurotoxins although their toxicity is at least an order of magnitude less. This naturally poses the question as to how such a difference in toxicity can arise in toxins with a high degree of homology. We considered that the determination of the primary structure of these cardiotoxins might reveal the significance of these observations as well as aid the identification of the functionally important amino acid positions.

This paper deals with the amino acid sequence of cardiotoxin which was obtained at a yield of about 16.4% from the crude venom of *Naja naja atra* and was the most abundant protein in the venom. In 1970 Narita and Lee [2] reported the complete amino acid sequence of cardiotoxin. However, our results revealed the presence of Leu–Val at position 48–49 and not Val–Leu as reported by Narita and Lee.

* To whom inquiries about this article should be directed.
** Himeji Institute of Technology, Himeji, Japan.

2. Materials and methods

Lyophilized *Naja naja atra* venom was obtained from Sigma Chemical Co., USA. The venom dissolved in 1% acetic acid was applied on a column of Sephadex G-50 equilibrated with the same medium. The protein fraction with a molecular weight of about 6000–7500 was freeze-dried and applied to a CM-cellulose column, equilibrated with a 0.005 M sodium acetate buffer, pH 5.8. Fractions were eluted with sodium acetate buffer which was applied to the column in the form of a linear gradient of concentration and pH (from 0.005 M, pH 5.8, to 0.5 M, pH 6.5). Each fraction was gel-filtered to remove sodium acetate, and the homogeneity was confirmed by disc gel electrophoresis. The third fraction which was cytotoxic to Yoshida sarcoma cells had an amino acid composition consistent with that of cardiotoxin. This fraction contained the major protein isolated from the crude venom and represented a yield of 16.4% of the original material. Its LD_{50} in mice (NIH strain) following intraperitoneal injection was determined according to the method of Litchfield and Wilcoxon [16]. Chick biventer cervicis muscle preparation [17] was used for testing the action on skeletal muscle.

The reduced and *S*-carboxymethylated (RCM) cardiotoxin was prepared by the method reported by Crestfield [18]. Amino acid analyses of cardiotoxin and the peptides generated by cyanogen bromide cleavage or tryptic digestion of the RCM-toxin were performed with the standard method using an Hitachi KLA 3B amino acid analyzer. Sequence analyses were conducted by the modified Edman procedure [19,20], and the phenylthiohydantoin amino acids were identified by thin layer chromatography employing several

North-Holland Publishing Company – Amsterdam

solvent systems [21–23]. Carboxypeptidase A digestion was performed according to standard methods [24]. Cyanogen bromide cleavage of the RCM-toxin was carried out in 70% formic acid by the method of Gross and Witkop [25]. The resulting peptides were fractionated on a column of Sephadex G-25 using 0.2 M acetic acid as an eluate and were further purified on a column of DEAE-cellulose. Tryptic peptides were separated by high voltage paper electrophoresis at pH 3.6, and paper chromatography in a solvent system of 1-butanol–acetic acid–water (4:1:5, by vol), water-saturated phenol, or 1-butanol–acetic acid–pyridine–water (15:3:10:12).

3. Results and discussion

By the CM-cellulose chromatography of the fraction No. IV obtained by gel filtration of the venom of *Naja naja atra* using Sephadex G-50, at least four kinds of cardiotoxin-like components were fractionated in an homogeneous state by disc gel electrophoresis. Cardiotoxin was the most abundant protein which was isolated from the venom of *Naja naja atra*. The LD_{50} of cardiotoxin in mice after intraperitoneal injection was estimated to be 68 (65 to 70) μg/g body weight. The mol. wt. was estimated by gel filtration to be about 7000. Based on this value and on the amino acid analysis, one molecule of cardiotoxin contains about 60 amino acid residues: Asp 5.69, Thr 2.76, Ser 1.96, Pro 5.30, Gly 1.75, Ala 1.79, Cys 6.51, Val 5.94, Met 1.70, Ile 1.0, Leu 5.48, Tyr 2.55, Phe 1.93, Lys 7.40, Arg 2.04.

Thirty two stepwise Edman degradation of RCM-cardiotoxin revealed the amino-terminal sequence to be: H-Leu–Lys–Cys–Asn–Lys–Leu–Val–Pro–Leu–Phe–Tyr–Lys–Thr–Cys–Pro–Ala–Gly–Lys–Asn–Leu–Cys–Tyr–Lys–Met–Phe–Met–Val–Ala–Thr–Pro–Lys–Val ....

The carboxy-terminal fragment CB-III, obtained by the cyanogen bromide cleavage of the RCM-toxin, in which homoserine was absent, contained 34 amino

Table 1
Amino acid composition of cardiotoxin and peptides generated by CNBr cleavage

Amino acid	Cartiotoxin	CNBr fragments CB-I	CB-II	CB-III
CM-Cysteine	–	1.7(3)		3.1(5)
Aspartic acid	6.6(6)	2.5(2)		4.1(4)
Threonine	2.9(3)	1.1(1)		1.7(2)
Serine	2.7(2)	0.6(0)		1.6(2)
Glutamic acid	0	0		0
Proline	5.8(5)	2.1(2)		2.1(3)
Glycine	2.3(2)	1.0(1)		1.0(1)
Alanine	1.8(2)	0.9(1)		0.9(1)
Valine	4.6(7)	1.4(1)		3.8(6)
Methionine	2.0(2)	0		0
Isoleucine	0.9(1)	0		0.7(1)
Leucine	5.7(6)	3.1(4)		2.1(2)
Tyrosine	2.7(3)	1.5(2)		1.0(1)
Phenylalanine	2.1(2)	1.2(1)	1.0(1)	0.5(0)
Tryptophan	0	0		0
Half-Cystine	7.7(8)	0		0
Lysine	7.9(9)	4.0(5)		3.4(4)
Histidine	0	0		0
Homoserine	–	0.9(1)	0.9(1)	0
Arginine	2.0(2)	0.5(0)		1.6(2)

Numbers in parentheses represent the values to the nearest integer. In cardiotoxin, the value of arginine was taken as 2.0, in CB-I and CB-III, the value of glycine was taken as 1.0, and in fragment CB-II, the value of phenylalanine was taken as 1.0.

Table 2
Amino acid composition of tryptic peptides of CB-I of cardiotoxin

Amino Acid	T-1	T-2	T-2′	T-3	T-4	T-5	T-6	CB-I
CM-Cystein				1.0(1)	1.0(1)	1.0(1)		3
Aspartic acid				1.1(1)		1.1(1)		2
Threonine					0.9(1)			1
Serine								0
Glutamic acid								0
Proline			1.0(1)		1.1(1)			2
Glycine					1.0(1)			1
Alanine					1.0(1)			1
Valine			0.9(1)					1
Methionine								0
Isoleucine								0
Leucine	1.0(1)		2.0(2)	1.1(1)				4
Tyrosine		0.8(1)		1.1(1)				2
Phenylalanine		0.9(1)						1
Tryptophan								0
Half-Cystine								0
Lysine	1.0(1)	1.0(1)		1.0(1)	1.0(1)	1.0(1)		5
Histidine								0
Homoserine							1.0(1)	1
Arginine								0

In each column the values of the amino acids underlined were taken as 1.0. The numbers in parentheses represent the values to the nearest integer.

Table 3
Amino acid composition of tryptic peptides of CB-III of cardiotoxin

Amino acid	T′-1	T′-1′	T′-2	T′-3	T′4	T′5	T′6	CB-III
CM-Cysteine					2.0(2)	2.5(2)	1.0(1)	5
Aspartic acid					1.3(1)	2.6(2)	1.2(1)	4
Threonine			1.2(1)			1.0(1)		2
Serine				2.0(2)				2
Glutamic acid								0
Proline	1.0(1)		1.3(1)		1.1(1)			3
Glycine					1.2(1)			1
Alanine			1.2(1)					1
Valine	1.8(2)		1.6(1)	1.0(1)	1.0(1)	1.3(1)		6
Methionine								0
Isoleucine					1.0(1)			1
Leucine				2.4(2)				2
Tyrosine						0.8(1)		1
Phenylalanine								0
Tryptophan								0
Lysine	1.0(1)		1.0(1)	1.0(1)	1.0(1)			4
Homoserine								0
Arginine		1.0(1)				1.0(1)		2

In each column the values of the amino acid underlined were taken as 1.0. The numbers in parentheses represent the values to the nearest integer.

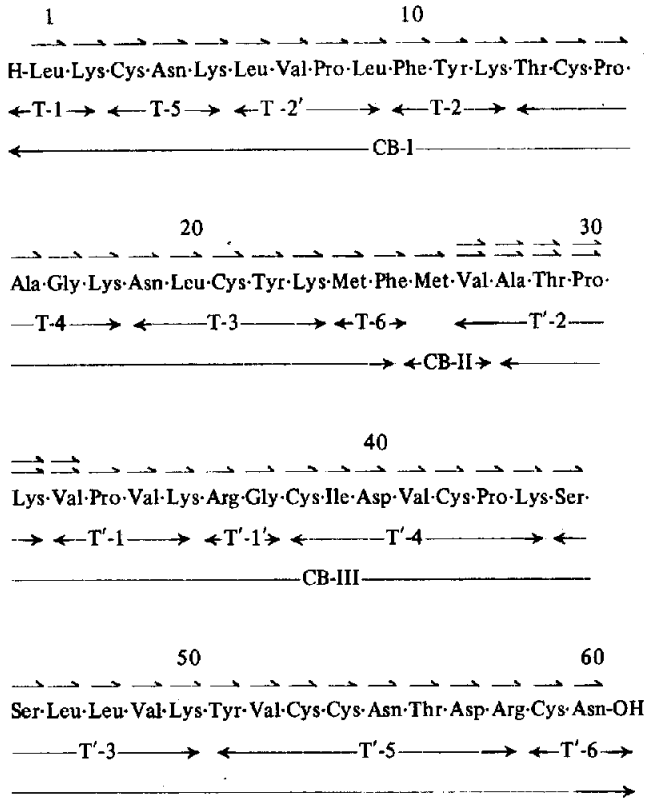

Fig.1. Amino acid sequence of cardiotoxin from Formosan cobra venom (*Naja naja atra*). Horizontal arrows above and below amino acid residues denote the sequence of CNBr fragments derived from RCM-toxin and tryptic peptides. Right- and left-handed arrows show that the sequence was elucidated, respectively, by Edman degradation or by the action of carboxypeptidase A. T and T′ represent the peptides produced by tryptic digestion of fragments CB-I and CB-III, respectively.

acid residues. Stepwise Edman degradation gave all of the amino acid residues, v.s. –Val–Ala–Thr–Pro–Lys–Val–Pro–Val–Lys–Arg–Gly–Cys–Ile–Asp–Val–Cys–Pro–Lys–Ser–Ser–Leu–Leu–Val–Lys–Tyr–Val–Cys–Cys–Asn–Thr–Asp–Arg–Cys–Asn-OH.

The carboxy-terminal sequence was then examined by the use of carboxypeptidase A. After 24 h incubation, aspargine and carboxymethylcysteine were liberated on the ratio of Asn:Cys(CM)=1.0:0.5. From this result, the carboxy-terminal sequence was considered to be –Cys–Asn–Asn-OH. To ascertain the carboxy-terminal sequence, the RCM-toxin was digested with trypsin and the product was found to be a peptide consisting of Asp:Cys(Cm)=1.0:0.8. From these results, the cartoxy-terminal sequence was determined to be –Cys–Asn-OH.

The sequence was further ascertained by separation of the tryptic peptides from the fragments derived from the cyanogen bromide cleavage of cardiotoxin. The RCM-toxin was cleaved by cyanogen bromide in 70% formic acid for 24 h at 37°C and the resulting peptide fragments were fractionated by gel filtration on a column of Sephadex G-25. High mol. wt. fragments CB-I and CB-III were further purified by chromatography on a column of DEAE-cellulose. The low mol. wt. fragment CB-II, which appeared in the column volume of gel filtration procedure, was

further purified by paper electrophoresis and paper chromatography.

The amino acid compositions of these peptides are given in table 1. The fragments CB-I and CB-III, generated from the cyanogen bromide cleavage of the RCM-toxin were digested by trypsin and the resulting peptides were separated by high voltage paper electrophoresis and paper chromatography. The amino acid compositions of the peptides were determined by amino acid analyses and are shown in tables 2 and 3. The central fragment CB-II, which contained homoserine and was located between the amino- and carboxy-terminal fragments, was determined by phenylalanyl homoserine. On the basis of the above results, the amino acid sequence of cardiotoxin was found to be CB-I-II-III.

The primary sequence of cardiotoxin can now be expressed as shown in fig.1. The amino acid sequence differs from that of cardiotoxin reported by Narita

48 49 48 49

and Lee in having Leu–Val in place of Val–Leu. However, a reinvestigation of the sequence of their tryptic peptide corresponding to the present T′-3 by Narita [26] has led to a revision to the sequence which now is in agreement with that determined in this investigation.

Acknowledgements

The authors are indebted to Professor Ikuo Yamashina of our laboratory for valuable discussions and encouragement in the course of the study and also to Mrs Kiyoe Ohta for skillful technical assistance. The authors are also indebted to Dr. B. Witkop of the National Institutes of Health for his help in preparing this manuscript. This work was aided in part by a Grant from the Ministry of Education.

References

[1] Larsen, P. R. and Wolf, J. (1968) J. Biol. Chem. 243, 1283–1296.
[2] Narita, K. and Lee, C. Y. (1970) Biochem. Biophys. Res. Commun. 41, 339–343.
[3] Hayashi, K., Takechi, M. and Sasaki, T. (1971) Biochem. Biophys. Res. Commun. 45, 1357–1362.
[4] Takechi, M., Hayashi, K. and Sasaki, T. (1973) Molecular Pharmacol. 8, 446–451.
[5] Hayashi, K., Takechi, M., Sasaki, T. and Lee, C. Y. (1975) Biochem. Biophys. Res. Commun. 64, 360–366.
[6] Louw, A. I. (1974) Biochim. Biophys. Acta 336, 481–495.
[7] Louw, A. I. (1974) Biochem. Biophys. Res. Commun. 58, 1022–1029.
[8] Fryklund, L. and Eaker, D. (1973) Biochemistry 12, 661–667.
[9] Carlsson, F. H. H. (1974) Biochem. Biophys. Res. Commun. 59, 269–276.
[10] Carlsson, F. H. H. and Joubert, F. J. (1974) Biochim. Biophys. Acta 336, 453–469.
[11] Weise, K. H. K., Carlsson, F. H. H. Joubert, F. J. and Strydom, D. J. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 1317–1326.
[12] Takechi, M., Sasaki, T. and Hayashi, K. (1973) Proceedings of the Symposium on 11th Peptide Chemistry p. 108.
[13] Fryklund, L. and Eaker, D. (1975) Biochemistry 14, 2860–2865.
[14] Fryklund, L. and Eaker, D. (1975) Biochemistry 14, 2865–2871.
[15] Shipolini, R. A., Kissonerghis, M. and Banks, B. E. C. (1975) Eur. J. Biochem. 56, 449–454.
[16] Litchfield, J. T. and Wilcoxon, F. (1948) J. Pharmacol. Exp. Ther. 96, 99–113.
[17] Ginsborg, B. L. and Warriner, J. (1960) Brit. J. Pharmacol. 15, 410–411.
[18] Crestfield, A. M., Moore, S. and Stein, W. H. (1963) J. Biol. Chem. 238, 622–627.
[19] Niall, H. D., Keutmann, H. T., Copp, D. H. and Potts, Jr. J. T. (1969) Proc. Nat. Acad. Sci. 64, 771–778.
[20] Iwanaga, S., Wallen, P., Gröndahl, N. J., Henschen, A. and Blömback, B. (1969) Eur. J. Biochem. 8, 189–199.
[21] Brenner, M., Niederwieser, A. and Pateke, G. (1961) Experientia 17, 145–153.
[22] Jeppsson, J. O. and Sjöquist, J. (1969) Anal. Biochem. 18, 264–269.
[23] Morgan, F. J. and Henschen, A. (1969) Biochim. Biophys. Acta 181, 93–104.
[24] Ambler, R. P. (1967) Methods in Enzymology (ed. by C. H. W. Hirs) Vol. 11, 436–445, Academic Press, New York, London.
[25] Gross, E. and Witkop, B. (1967) Biochemistry 6, 745–748.
[26] Personal communication.