Unusual amino acid sequence of fasciatoxin, a weak reversibly acting neurotoxin in the venom of the banded krait, *Bungarus fasciatus*

Chen-Sheng LIU,*† Po-Wen HSIAO,* Chen-Shying CHANG,* Mu-Chin TZENG*† and Tung-Bin LO*†

*Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan, and †Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan, Republic of China

A weak reversibly acting neurotoxin, fasciatoxin, was found in the venom of *Bungarus fasciatus*. The sequencing was completed by manual and automated Edman analyses of the reduced and carboxymethylated protein and of the peptides obtained from enzyme digestions. It is composed of 63 amino acid residues with four disulphide bonds and a unique sequence at the C-terminal end. According to the criteria set by Ryden, Gabel & Eaker [(1973) *Int. J. Pept. Protein Res.* 5, 261–273], fasciatoxin lacks all of the five functionally invariant residues of neurotoxins. The hydropathy index indicates that fasciatoxin is devoid of a strong hydrophilicity domain for binding to the receptor site. Structural comparison with some typical neurotoxins also reveals the uniqueness of fasciatoxin in that the extent of similarity is only about 30%.

INTRODUCTION

Snake venom provides a rich source of biologically active substances such as neurotoxins, cardiotoxins, diverse enzymes, enzyme inhibitors and some other factors. Among them, the most studied neurotoxins generally fall into two groups: short-chain neurotoxins of 60–62 amino acid residues with four disulphide bonds, and long-chain neurotoxins of 70–74 amino acid residues with five disulphide bonds. Irrespective of the molecular size, these toxins bind very tightly, and in most cases irreversibly, to the nicotinic acetylcholine receptors at the neuromuscular junction (Lee *et al.*, 1972; Harris, 1984). However, to our knowledge, there are two structurally characterized neurotoxins that are weak reversibly acting neuromuscular blocking agents. They are toxin LsIII from *Laticauda semifasciata* (66 amino acid residues with five disulphide bonds) (Maeda & Tamiya, 1974; Harvey & Rodger, 1978) and pseudonajatoxin b from *Pseudonaja textilis* (71 amino acid residues with five disulphide bonds) (Tyler *et al.*, 1987).

During investigation of the venom of *Bungarus fasciatus*, our laboratory has chemically characterized three analogous cardiotoxin-like substances (Lu & Lo, 1980, 1981), which are the major constituents of the venom, and two analogous proteinase inhibitors (Liu *et al.*, 1983).

Subsequently the neurotoxic material was found to be located in fraction VII in the CM-cellulose chromatography profile (Liu *et al.*, 1981), and further purification by h.p.l.c. revealed two major peaks. Sequencing work on the first component (VII-1) resulted in a typical short-chain neurotoxin comprising 64 amino acid residues with four disulphide bridges (Liu *et al.*, 1989).

The present work describes the purification, amino acid sequence determination and pharmacological characterization of the second component (VII-2), which

is now named 'fasciatoxin' in view of its unusual amino acid sequence, distinctly different from other known short-chain-type toxins, including neurotoxins and cardiotoxins.

MATERIALS AND METHODS

Materials

Crude venom was obtained from Miami Serpentarium Laboratories, Salt Lake City, UT, U.S.A. Clostripain was obtained from Sigma Chemical Co., and lysyl endopeptidase from Wako Chemicals. CM-cellulose (CM-52) was obtained from Whatman. Phenyl isothiocyanate and dimethylaminoazobenzene isothiocyanate were the products of Pierce Chemical Co. All other chemicals and solvents used in h.p.l.c. separation and amino acid sequencing were obtained from commercial sources.

Isolation and purification

Fraction VII was obtained from CM-cellulose chromatography of the crude venom as described previously (Liu *et al.*, 1981), and fraction VII-2 was obtained after further fractionation by h.p.l.c. (see Fig. 1). The M_r was determined by the gel-filtration method of Andrews (1964).

Assay of neurotoxicity

The toxicity test to determine LD₅₀ was carried out by the method of Lichtfield & Wilcoxon (1949). Measurement of neurotoxicity was done by a bioassay using the biventer cervicis nerve-muscle preparation of the chick (Ginsborg & Warriner, 1960). The muscles of 4–6-day-old Leghorn chicks were suspended in 10 ml of Tyrode's solution (composition: 137 mM-NaCl, 2.7 mM-KCl, 2.8 mM-CaCl₂, 1.0 mM-MgCl₂, 0.36 mM-NaH₂PO₄, 12 mM-

Abbreviation used: RCM-, reduced and carboxymethylated.

† To whom requests for reprints should be addressed.

These sequence data have been submitted to the EMBL/GenBank Data Libraries.

NaHCO₃ and 11 mM-glucose) constantly gassed with O₂/CO₂ (19:1) at 37 °C. The nerve was stimulated with supramaximal rectangular pulses of 0.05 ms duration at 5 s intervals. The muscle tension was measured isometrically with Grass FT.03 force-displacement transducers and recorded with a model 79 polygraph.

Separation of enzymic digests

Carboxymethylation of the protein under reducing conditions was carried out essentially by the procedure of Crestfield *et al.* (1963). Clostripain digests were obtained by incubating the RCM-protein with the enzyme (100:1, w/w) in 50 mM-NH₄HCO₃ containing 5 mM-dithiothreitol at 37 °C for 3 h. Similarly, lysyl endopeptidase digestion (30:1, w/w) took place in 20 mM-Tris/HCl buffer, pH 8.7, at 37 °C for 3 h. Enzymic digests were directly injected on to the h.p.l.c. system, which employed a Vydac C4 column and Nucleosil C₁₈ column with solvent A (0.07% trifluoroacetic acid in water) and solvent B (0.07% trifluoroacetic acid in acetonitrile). This h.p.l.c. was essentially the Waters system.

Structural characterization

Amino acid analysis was conducted by a method that utilizes the hydrolysis conditions of 7 M-HCl/10% (v/v) trifluoroacetic acid/0.1% phenol at 158 °C for 30 min (Tsugita *et al.*, 1987) and subsequent reversed-phase h.p.l.c. of dimethylaminoazobenzenesulphonyl-amino acids (Knecht & Chang, 1986). Amino acid sequencing was performed either by manual operation of the dimethylaminoazobenzene isothiocyanate/phenyl

isothiocyanate method (Chang, 1983) or by using a gas-phase Sequenator (model 477A; Applied Biosystems) with an on-line amino acid phenylthiohydantoin h.p.l.c. system. Secondary-structure analysis in terms of hydrophobicity indexes was performed by the method of Kyte & Doolittle (1982) with a computerized program.

RESULTS

Purification of fasciatoxin (VII-2) from fraction VII in the CM-cellulose chromatography profile was achieved by h.p.l.c. as shown in Fig. 1. The yield of fasciatoxin is about 0.6% of the whole venom. Its *M_r* was found to be 7200 by gel filtration, and the actual *M_r* is 6847. Fasciatoxin expressed a weak lethality as revealed in an LD₅₀ of 8 µg/g body wt. of mouse when injected intraperitoneally. In contrast, the chromatographically adjacent neurotoxin, VII-1, showed an LD₅₀ of 0.17 µg/g body wt. of mouse.

As shown in Fig. 2, within 2 min after the application of fasciatoxin to give a final concentration of 5 µg/ml in the solution bathing a biventer cervicis nerve-muscle preparation of the chick, the muscle-twitch response to indirect stimulation began to decrease gradually, and was completely abolished after about 20 min. After the blockade of neuromuscular transmission, the muscle response to 5 µg of exogenous acetylcholine/ml was also inhibited. Muscle contraction was not seen at this dose of toxin and even at a concentration as high as 50 µg/ml. The blocking effect of the toxin on the twitch response and that on the response to exogenous acetylcholine were both reversed by a single wash with Tyrode's solution, with both responses returning to near normal about 1 h after the wash.

The h.p.l.c. profiles of clostripain digests and lysyl endopeptidase digests are shown in Figs. 3 and 4 respectively. There are three major clostripain peaks (Cl-1–Cl-3) and six lysyl endopeptidase peaks (LE-1–LE-6). On rechromatography, LE-2 gave a major peak (LE-2-1) preceding and a minor subsequent peak (LE-2-2) with peak area ratio of 5.7:1 (results not shown). The results of amino acid analyses of the intact protein as well as three clostripain digests are compiled in Table 1. The numbers of individual amino acid residues totalled from the data for the clostripain digests are in complete agreement with those in the intact protein. The whole molecule has 63 amino acid residues and is devoid of tyrosine. Similarly, Table 2 assembles those data obtained from the lysyl endopeptidase digestion. The analysis result for peptide LE-2-2 was equivalent to that for peptide LE-3. Peptide LE-6 contains the residues of both

Fig. 1. Purification of fasciatoxin by h.p.l.c.

About 0.2 mg of fraction VII was applied to the Vydac C4 column and eluted with a linear gradient of 10–60% (v/v) solvent B (0.07% trifluoroacetic acid in acetonitrile) over 70 min at a flow rate of 1 ml/min. Absorbance full scale was 1.0 and was monitored at 214 nm.

Fig. 2. Effect of fasciatoxin on the chick biventer cervicis

The biventer cervicis nerve-muscle preparation from a male Leghorn chick was incubated at 37 °C in 10 ml of modified Tyrode's solution. To monitor neuromuscular transmission, the nerve of the biventer cervicis was stimulated with supramaximal pulses (0.05 ms at 5 s intervals). Electrical stimulation was interrupted during testing of response to 5 µg of acetylcholine/ml (A). The time of application of fasciatoxin (final concentration 5 µg/ml) is indicated by the arrow. At point W the preparation was washed with the Tyrode's solution. Calibrations: 10 min and 1 g.

peptide LE-4 and peptide LE-5. After all the analyses, only a tripeptide (CM-Cys-His-Lys) is not accounted for, and it is assumed that this hydrophilic peptide appeared in the most unretarded peak, which preceded peak LE-1. The presence of one-twelfth amounts of both carboxy-

methyl-cysteine and histidine in peak LE-1 supports the above proposition.

Manual sequencing and automatic sequencing complement each other to produce the entire amino acid sequence (Fig. 5a). The *N*-terminal 23 amino acid residues from the Sequenator work is illustrated in Fig. 5(b), and the results for the enzymic peptides in Figs. 5(c) and 5(d). Automatic sequencing was carried out twice for peptide LE-3 to confirm the result. Alignment order was readily determined by the three clostripain peptides, since both *N*-terminal and intermediate peptides were terminated at an arginine residue. The preponderance of basic amino acid residues indicates that fasciatoxin is a very basic neurotoxin.

DISCUSSION

Fasciatoxin blocks both the muscle-twitch response to nerve stimulation and the response to exogenous acetylcholine without causing muscle contraction, and thus it acts in the same manner as the curaremimetics and belongs to the group of post-synaptic neurotoxins from snake venoms. However, fasciatoxin is uncommon in some aspects. The neuromuscular blockade caused by most of the post-synaptic neurotoxins, both short-chain and long-chain, on the chick muscle cannot be reversed by repeated washes. In contrast, the effect of fasciatoxin is readily reversible. To date, only toxin LsIII has also been established to act reversibly on the chick muscle (Harvey & Rodger, 1978; Maeda *et al.*, 1974). Surprisingly, the amino acid sequence of fasciatoxin shows no more homology to that of toxin LsIII than to those of other toxins in the same group (see below). The potency of fasciatoxin on the chick muscle is about twice that of toxin LsIII, which caused complete neuromuscular blockade after about 20 min at 10 µg/ml. In contrast, the highly potent neurotoxins cobrotoxin

Fig. 3. Separation of clostripain digest of RCM-fasciatoxin by h.p.l.c.

About 0.3 mg of clostripain digest of RCM-fasciatoxin was applied to Vydac C4 column and eluted with a linear gradient of 5–65% (v/v) solvent B over 1 h. The other conditions are the same as indicated in Fig. 1 legend.

Table 1. Amino acid compositions of the intact fasciatoxin and clostripain peptides of RCM-fasciatoxin

Abbreviation: CM-Cys, carboxymethyl-cysteine.

	Amino acid composition (mol of residue/mol)				
	From sequence	Intact protein	Peptide CI-1	Peptide CI-2	Peptide CI-3
Asx	4	4.4	1.9 (2)	1.4 (1)	1.4 (1)
Glx	5	4.8			5.1 (5)
CM-Cys			4.7 (5)		2.6 (3)
Ser	6	6.4	3.3 (4)	2.0 (2)	
Thr	6	6.0	3.7 (4)		1.9 (2)
Gly	4	4.9	4.2 (4)		
Ala	3	3.5	1.7 (2)		1.3 (1)
Arg	2	2.5		1.3 (1)	1.4 (1)
Pro	5	5.0	1.8 (2)	2.5 (2)	1.2 (1)
Val	2	1.7		1.7 (2)	
Met	2	1.5	1.5 (1)	1.1 (1)	
Ile	2	1.7		0.6 (1)	1.4 (1)
Leu	4	3.9	1.0 (1)	1.0 (1)	1.8 (2)
Phe	2	2.3			2.0 (2)
CyS	8	7.7			
Lys	5	4.9	1.0 (1)	0.9 (1)	2.7 (3)
His	2	2.3		1.5 (1)	1.2 (1)
Trp	1	–			(1)

and α -bungarotoxin show the same blockade at about 0.6 $\mu\text{g/ml}$ and 1.5 $\mu\text{g/ml}$ respectively (Lee *et al.*, 1972; Maeda *et al.*, 1974).

Clostripain splits at two Arg-Xaa bonds and generates

Fig. 4. Separation of lysyl endopeptidase digest of RCM-fasciatoxin by h.p.l.c.

About 0.25 mg of digest was applied to Nucleosil C_{18} column and a linear gradient of 10–50% (v/v) solvent B over 120 min was used. Absorbance full scale was 2.0 and was monitored at 220 nm.

three major peptides (Fig. 3). Lysyl endopeptidase generally cleaves Lys-Xaa bonds and even a Lys-Pro bond in the present case.

As mentioned above, the neurotoxicity of fasciatoxin is relatively weak and readily reversible. Sequence homology with other closely related toxins, one neurotoxin from *Naja mossambica mossambica*, one cardiotoxin from *Naja haje haje*, κ -bungarotoxin from *Bungarus multicinctus* and toxin LsIII from *Laticauda semifasciata*, are compared to reveal the degree of relatedness (Fig. 6). More deletion gaps were placed for fasciatoxin in order to arrange half-cysteine residues in the proper positions. Fasciatoxin displays 33% similarity with the neurotoxin and 36% similarity with the cardiotoxin, and its structure lies somewhere between those of the two types of toxins. The same situation occurs in the sea-snake toxin LsIII (Maeda & Tamiya, 1974). Sequence comparisons of fasciatoxin with other typical short-chain neurotoxins such as cobrotoxin of *Naja naja atra*, toxin S_5C_1 of *Dendroaspis jamesoni kaimosae*, toxin b of *Aipysurus laevis*, toxin c of *Laticauda colubrina* and the functionally related toxin LsIII resulted in 32%, 24%, 29%, 24% and 27% similarities whereas 39% similarity was found between neurotoxin I of *Naja mossambica mossambica* and toxin LsIII. Grant and co-workers have reported a new family of snake-venom neurotoxins present only in *Bungarus* species (Grant & Chiappinelli, 1985; Grant *et al.*, 1988). They are κ -bungarotoxin and κ -flavitoxin obtained respectively from the venoms of *Bungarus multicinctus* and *Bungarus flaviceps*. Both toxins bind more specifically to neuronal nicotinic receptors, and when compared with α -neurotoxins they bind to muscle nicotinic receptors with much lower affinity (Wolf *et al.*, 1988). They both have 66 amino acid residues with five disulphide bonds. When κ -bungarotoxin is aligned with fasciatoxin to seek the maximum degree of similarity, 30% similarity was found. Fasciatoxin is also unique in having a longer tail of -Cys-Asn-Gly-Pro-Ser-Thr-Pro-Ser-Thr, whereas with other short-chain toxins the C-terminals are usually

Table 2. Amino acid compositions of lysyl endopeptidase peptides of RCM-fasciatoxin

Abbreviation: CM-Cys, carboxymethyl-cysteine.

Amino acid composition (mol of residue/mol)					
Peptide LE-1	Peptide LE-2-1	Peptide LE-3	Peptide LE-4	Peptide LE-5	Peptide LE-6
Asx	1.2 (1)	1.9 (2)		1.3 (1)	1.0 (1)
Glx	3.2 (3)			2.2 (2)	2.1 (2)
CM-Cys	1.1 (1)	2.8 (3)	2.2 (2)	1.1 (1)	3.3 (3)
Ser		1.6 (2)	3.3 (4)		3.8 (4)
Thr	0.92 (1)	2.7 (3)	1.4 (1)	1.2 (1)	2.1 (2)
Gly		1.9 (2)	2.1 (2)		2.3 (2)
Ala	1.3 (1)	1.6 (2)			
Arg			0.80 (1)	1.0 (1)	2.0 (2)
Pro	1.0 (1)	1.9 (2)	1.9 (2)		2.2 (2)
Val			0.51 (1)	0.88 (1)	1.4 (2)
Met		1.0 (1)	1.0 (1)		1.2 (1)
Ile	0.86 (1)		0.51 (1)		0.68 (1)
Leu	0.95 (1)	0.86 (1)	0.93 (1)	1.0 (1)	2.1 (2)
Phe		1.0 (1)		1.0 (1)	1.4 (1)
Lys	1.0 (1)	0.84 (1)	1.0 (1)	0.81 (1)	2.0 (2)
His			0.75 (1)		0.91 (1)
Trp				(1)	(1)

Fig. 5. Results of amino acid sequencing of fasciatoxin

The amino acid sequence of fasciatoxin is shown in (a). For others see the explanation in the text.

Fig. 6. Sequence similarity of fasciatoxin (a) to *Naja mossambica mossambica* neurotoxin I (b), *Naja haje haje* CM-12 (c), κ -bungarotoxin (d) and toxin LsIII of *Laticauda semifasciata* (e)

Amino acid residues are aligned on the basis of half-cystine positions to give the maximum degree of similarity.

Fig. 7. Hydropathy profiles of fasciatoxin (a, —), *Naja mossambica mossambica* neurotoxin I (a, ----) and toxin LsIII (b)

Hydrophobicity values with six amino acid residues clustered were calculated according to the procedure of Kyte & Doolittle (1982). See the text for explanation.

-Cys-Asn-Asn for the neurotoxins and -Cys-Asn for the cardiotoxins.

According to Ryden *et al.* (1973), there are 11 structurally invariant residues and five functionally invariant residues in both short-chain and long-chain neurotoxins. In *Naja mossambica mossambica* neurotoxin I the structurally invariant residues are Cys-3, -17, -24, -45, -49, -61, -62 and -65, Tyr-25, Gly-44 and Pro-50 and the functionally invariant residues are Lys-27, Trp-29, Asp-31, Arg-36 and Gly-37 (cf. Fig. 6). The latter proposition is in line with the conclusion obtained from the X-ray-crystallography study by Low *et al.* (1976) on erabutoxin b. A three-dimensional structure of a neurotoxin from a sea snake revealed six 'toxic' residues including the above-mentioned five residues plus Lys-53 (Tsernoglou & Petsko, 1977), and the relevant study on the long-chain neurotoxin from *Naja naja siamensis* indicated that the five residues, Trp-29, Asp-31, Arg-36, Gly-37 and Lys-53, are involved in the binding of the neurotoxin to acetylcholine receptors (Walkinshaw *et al.*, 1980). Assuming the propriety of alignment of similar residues, fasciatoxin appears to lack structurally invariant Tyr-25, Pro-50 and all of the five functionally invariant residues, which were replaced by Arg-27, Val-29, Pro-31, Pro-36 and Ser-37. It is not easy to assess the contribution of these substitute amino acids to the functionality, i.e. neurotoxicity, of fasciatoxin.

The hydropathy profiles (Figs. 7a and 7b) showed some interesting features among three toxins. The gross contour of the hydropathy profile for fasciatoxin looks more like that of toxin LsIII, and this feature may reflect their functional similarity. The hydropathy profiles for fasciatoxin and for the neurotoxin were superimposed to examine their difference(s) more closely. Fasciatoxin is clearly more hydrophobic, and a remarkable difference is seen in the area belonging to the second disulphide loop (the section between Cys-24 and Cys-42), which is considered to contain the recognition site for the receptor. The greatest hydrophilicity of this area in the true neurotoxin seems to attest the above proposition, and the much less hydrophilicity of fasciatoxin around this area may explain why there is only a weak reversible binding of the toxin with the receptor.

We are indebted to Mr. S.-W. Chen for his assistance with the gas-phase Sequenator and to Mr. J.-H. Yang in the neurotoxicity bioassay. We thank Ms. M.-C. Chen for her

secretarial assistance. The research was supported in part by the National Science Council, Republic of China.

REFERENCES

- Andrews, P. (1964) *Biochem. J.* **91**, 222-233
- Chang, J. Y. (1983) *Methods Enzymol.* **91**, 455-466
- Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622-627
- Ginsborg, B. L. & Warriner, J. (1960) *Br. J. Pharmacol.* **15**, 410-411
- Grant, G. A. & Chiappinelli, V. A. (1985) *Biochemistry* **24**, 1532-1537
- Grant, G. A., Frazier, M. W. & Chiappinelli, V. A. (1988) *Biochemistry* **27**, 3794-3798
- Harris, J. B. (1984) *Prog. Med. Chem.* **21**, 64-110
- Harvey, A. L. & Rodger, I. W. (1978) *Toxicon* **16**, 219-225
- Knecht, R. & Chang, J.-Y. (1986) *Anal. Chem.* **58**, 2375-2379
- Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105-132
- Lee, C.-Y., Chang, C.-C. & Chen, Y.-M. (1972) *T'ai-wan I Hsueh Hui Tsa Chih* **71**, 344-349
- Lichtfield, J. T., Jr. & Wilcoxon, F. (1949) *J. Pharmacol. Exp. Ther.* **96**, 99-113
- Liu, C.-S., Ho, C.-L., Lee, M.-T., Wu, T.-C. & Lo, T.-B. (1981) *Proc. Natl. Sci. Coun. Repub. China* **5**, 355-360
- Liu, C.-S., Wu, T.-C. & Lo, T.-B. (1983) *Int. J. Pept. Protein Res.* **21**, 209-215
- Liu, C.-S., Chen, J.-P., Chang, C.-S. & Lo, T.-B. (1989) *J. Biochem. (Tokyo)* **105**, 93-97
- Low, B. W., Preston, H. S., Sato, A., Rosen, L. S., Searl, J. E., Rudko, A. D. & Richardson, J. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2991-2994
- Lu, H.-S. & Lo, T.-B. (1980) *Proc. Natl. Sci. Coun. Repub. China* **4**, 41-54
- Lu, H.-S. & Lo, T.-B. (1981) *Toxicon* **19**, 103-111
- Maeda, N. & Tamiya, N. (1974) *Biochem. J.* **141**, 389-400
- Maeda, N., Takagi, K., Tamiya, N., Chen, Y.-M. & Lee, C.-Y. (1974) *Biochem. J.* **141**, 383-387
- Ryden, L., Gabel, D. & Eaker, D. (1973) *Int. J. Pept. Protein Res.* **5**, 261-273
- Tsernoglou, D. & Petsko, G. A. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 971-974
- Tsugita, A., Uchida, T., Mews, H. W. & Ataka, T. (1987) *J. Biochem. (Tokyo)* **102**, 1593-1597
- Tyler, M. I., Spence, I., Barnett, D. & Howden, M. E. H. (1987) *Eur. J. Biochem.* **166**, 139-143
- Walkinshaw, M. D., Saenger, W. & Mealicke, A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2400-2404
- Wolf, K. M., Ciarleglio, A. & Chiappinelli, V. A. (1988) *Brain Res.* **439**, 249-258