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The Isolation of an Easily Reversible Post-Synaptic Toxin from the Venom of a Sea Snake, *Laticauda semifasciata*

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A weakly neurotoxic component (Ls-III) was isolated by CM-cellulose column chromatography from the venom of a sea snake *Laticauda semifasciata*. The content of component LsIII was about 10–20% of the venom as determined by u.v. absorption at 280nm. Component LsIII was homogeneous on rechromatography and disc electrophoresis, and its molecular weight was shown to be 7100 by ultracentrifugation and 7300 by sodium dodecyl sulphate–polyacrylamide-gel disc electrophoresis. The isoelectric point of component LsIII was pH 7.2. Component LsIII consisted of 66 amino acid residues including 10 half-cystine residues. The LD_{50} of component LsIII by intramuscular injection was 1.24μg/g body wt. for mice and 0.45μg/g for baby chicks, which is about eight to ten times less toxic than erabutoxins a, b and c, all of which are contained in the same venom. Experiments with three isolated muscle preparations from different species indicated that component LsIII was a post-synaptically acting toxin, the action of which was easily reversed by washing.

Three neurotoxins, erabutoxins a, b and c, were isolated and sequenced from the venom of the sea snake *Laticauda semifasciata* (Tamiya & Arai, 1966; Tamiya & Abe, 1972; Sato & Tamiya, 1971; Endo *et al.*, 1971). More than 95% of the lethal activity of the venom was ascribed to these toxins. A new less-toxic component was isolated from the same venom. The present paper describes the isolation and some properties of the component, which was named Laticauda semifasciata III (component LsIII).

Materials and Methods

Sea-snake venom

The sea snakes *Laticauda semifasciata* were collected at Amami Islands and Miyako Islands, Japan. The snake heads were kept frozen until use. Venom was extracted by the method of Tamiya & Arai (1966).

Column chromatography

The elution of proteins from the column was followed by measuring the extinction at 280nm continuously by using a Toyo Uvicon 540M photometer (Toyo Scientific Instruments Co., Tokyo, Japan).

Disc electrophoresis

The disc electrophoresis of the venom components was carried out at pH4.0 (Reisfeld *et al.*, 1962) with the slight modifications of Tamiya & Arai (1966).

Sodium dodecyl sulphate–polyacrylamide-gel disc electrophoresis (Weber & Osborn, 1969) was carried out in 0.1% sodium dodecyl sulphate in 10% (w/v) polyacrylamide gel. Each sample (0.5mg) was incubated overnight at 37°C with 1% sodium dodecyl sulphate in 50% (v/v) glycerol in 0.01M-sodium phosphate buffer, pH7.0, containing 1% (v/v) 2-mercaptoethanol (1.0ml). The sample solution was applied (0.1ml/gel) and electrophoresis performed at a constant current of 8mA/gel for 5h.

Ultracentrifugation

Component LsIII was dissolved in 0.05M-sodium–potassium phosphate buffer, pH7.0, containing 0.1M-NaCl. A solution of E_{280}^{1cm} 17.5 (8.5mg/ml) was subjected to the approach-to-equilibrium sedimentation method of Archibald (Schachman, 1959) in a cell described by Yphantis (1960).

Isoelectric focusing

The electrofocusing experiments were carried out in LKB 8100 Ampholine electrofocusing equipment according to the manual (LKB Produkter AB, Bromma, Sweden) at pH3–10 in a total volume of 100ml with the cathode at the top at 300V for 61h. Fractions (2g) were collected and measured for the extinction at 280nm and for pH values with a Hitachi 124 spectrophotometer (Hitachi Co., Tokyo, Japan) and with a TOA Dempa HM6A pH meter (TOA Dempa, Tokyo, Japan) respectively.

Amino acid analysis

Samples (0.5–1.0mg) of component LsIII and reduced and *S*-carboxymethylated component LsIII (Crestfield *et al.*, 1963; Maeda & Tamiya, 1974) were hydrolysed with 6M-HCl (0.4ml) in sealed glass tubes *in vacuo* at 105°C for 24, 48 and 72h. The analysis was performed on an automatic amino acid analyser (type JLC-5AH; Japan Electron Optics Laboratory Co., Tokyo, Japan). The tryptophan content was determined by u.v. absorption as described by Goodwin & Morton (1946).

Measurement of lethal activity

The lethal activity of component LsIII was assayed in both mice (NIH strain; 18–22g) and baby chicks (26–40g) by intramuscular injection of 0.1–0.2ml of a solution (the concentration was changed to contain the lethal dose in 0.1–0.2ml) in 0.85% NaCl. The LD_{50} was computed by the method of Litchfield & Wilcoxon (1949).

Rectus abdominis muscle preparation of the frog

The action of component LsIII on the isolated rectus abdominis muscle of the frog *Rana nigromaculata* was studied as described by Tamiya & Arai (1966) with SBIT force-displacement transducer and multipurpose recorder RH-20 (Nihon Kohden Co., Tokyo, Japan).

Biventer cervicis muscle preparation of the chick

The isolated biventer cervicis muscle preparation (Ginsborg & Warriner, 1960) from chicks (4–7 days old) was suspended with a tension of 0.5g in 20ml of Krebs' solution (Krebs & Henseleit, 1932), which was bubbled continuously with O_2+CO_2 (95:5) at 37°C. Indirect stimuli were applied through the tendon at a rate of six per min with supramaximal rectangular pulses of 0.5ms. The isometric contractions were recorded with a Grass FT-03 force-displacement transducer and a Grass model 5 polygraph (Grass Medical Instruments, Quincy, Mass., U.S.A.).

Phrenic-nerve–diaphragm preparation of the rat

The phrenic-nerve–diaphragm preparation of the rat (Bülbring, 1946) was suspended with a tension of 2g in 20ml of Tyrode's solution (Tyrode, 1910), which was constantly aerated with O_2+CO_2 (95:5) at 37°C. Indirect and direct supramaximal stimuli were applied alternatively every 10s with rectangular pulses of 0.1 and 0.5ms respectively. The contractions were recorded isometrically as described for the chick biventer cervicis muscle preparation.

Results

Isolation of component LsIII

The elution of proteins of *Laticauda semifasciata* venom from a CM-cellulose column is shown in Fig. 1. The elution was started at a lower pH (5.9) than that described previously (Tamiya & Arai, 1966; Tamiya & Abe, 1972). In the experiment shown in Fig. 1, 12.6mg (19.9%) of component LsIII, 6.57mg (10.4%) of phospholipase A and erabutoxin c, 7.45mg (11.8%) of erabutoxin a and 16.5mg (26.1%) of erabutoxin b were obtained from four glands (63.2mg, 100%) as measured by the extinction at 280nm, assuming that a solution with $E_{280}^{1cm}=1.0$ contained 1mg of protein/ml. The pooled component LsIII fraction was desalted and concentrated by a Diaflo apparatus with UM-2 filter (Amicon Corp., Lexington, Mass., U.S.A.) in 0.1M-acetic acid and freeze-dried. The content of component LsIII in the venom was 10–20% as determined by absorption at 280nm. Rechromatography of component LsIII gave a single symmetrical elution peak.

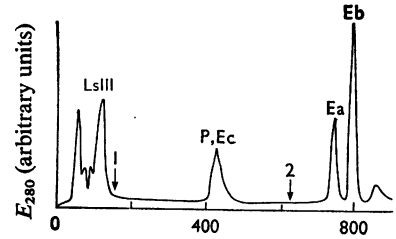

Fig. 1. *CM-cellulose column chromatography of Laticauda semifasciata venom*

Twelve glands (1.54g) of *L. semifasciata* were minced by scissors and extracted with 0.01M-sodium–potassium phosphate buffer, pH5.9 (15.4ml). The extract was dialysed in Visking cellulose tube and centrifuged. From the 15ml of the supernatant, 5ml was chromatographed on a CM-cellulose column (1.7cm×34cm), which had been equilibrated against the buffer. At arrow 1, the elution buffer was changed to 0.01M-phosphate buffer, pH6.4. At arrow 2, a linear gradient with NaCl concentration was applied, with 0.01M-phosphate buffer, pH6.4 (200ml) in the mixing chamber and the same buffer containing 0.2M-NaCl in the reservoir. LsIII, P, Ec, Ea and Eb represent component LsIII, phospholipase A, erabutoxin c, a and b, respectively.

Disc electrophoresis

The electrophoresis was performed at constant current of 3mA/gel [7.5% (w/v) acrylamide, pH4.0] for 2h with 40μg of sample. Component LsIII showed a single band which migrated to the cathode at a rate three-quarters of that of erabutoxin a.

Molecular-weight determination

Sodium dodecyl sulphate–polyacrylamide-gel disc electrophoresis. The molecular weight of component LsIII was estimated to be 7300 from the mobility in

Table 1. *Amino acid composition*

Results are expressed as mol of amino acid/mol of toxin. Values in parentheses are the nearest integers.

	Component LsIII	Reduced and *S*-carboxymethylated component LsIII		Erabutoxin a (Sato *et al.*, 1969)	Erabutoxin b (Sato *et al.*, 1969)	Erabutoxin c (Tamiya & Abe, 1972)
Lysine	3.96	3.95	(4)	4	4	3
Histidine	1.09	1.02	(1)	1	2	2
Arginine	2.10	1.81	(2)	3	3	3
Aspartic acid	6.25	6.18	(6)	5	4	5
Threonine‡	5.82	5.79	(6)	5	5	5
Serine‡	5.82	5.70	(6)	8	8	8
Glutamic acid	6.08	6.16	(6)	8	8	8
Proline	4.12	3.65	(4)	4	4	4
Glycine	4.53	4.15	(4)	5	5	5
Alanine	4.18	3.88	(4)	0	0	0
Cystine	8.65	8.00*	(10)	8	8	8
Valine§	3.01	2.91	(3)	2	2	2
Methionine	—	—	(0)	0	0	0
Isoleucine§	2.05	1.88	(2)	4	4	4
Leucine	2.15	1.95	(2)	1	1	1
Tyrosine	3.08	2.76	(3)	1	1	1
Phenylalanine	1.05	0.91	(1)	2	2	2
Tryptophan			(2)†	1	1	1
Total			66	62	62	62
Molecular weight						
Formula			7155	6838	6861	6847
Sodium dodecyl sulphate–polyacrylamide-gel-disc electrophoresis			7300			
Ultracentrifuge			7100	7430	7430	

* As *S*-carboxymethylcysteine: *S*-carboxymethylcysteine tends to give smaller values. The round number of 10 was confirmed by the sequence study (Maeda & Tamiya, 1974).
† Measured spectrophotometrically.
‡ Corrected for destruction.
§ Corrected for slow release.

ten repeated experiments, by using erabutoxin b, α-chymotrypsin, trypsin and pepsin as standards.

Ultracentrifugation. From the sedimentation curve of component LsIII obtained after centrifugation at 16.5°C and 20410rev./min, the molecular weight was calculated to be 7100.

Isoelectric point

Component LsIII was eluted as a single peak. The isoelectric point was pH7.2.

Amino acid composition

The results of amino acid analyses of 24h hydrolysates of component LsIII and its reduced and *S*-carboxymethylated derivative are given in Table 1. The values for serine and threonine or valine and isoleucine were confirmed by the results on 48 and 72h hydrolysates. The values in parentheses are also confirmed by sequence analysis (Maeda & Tamiya, 1974). The tryptophan/tyrosine ratio was 0.66. The formula weight obtained from the amino acid analyses agreed well with the molecular weight obtained by sodium dodecyl sulphate–polyacrylamide-gel disc electrophoresis and by ultracentrifugation. A solution of 1mg of component LsIII/ml had E_{280}^{1cm} value of 2.06, reflecting its high tryptophan and tyrosine content.

Lethal activity in mice

The LD_{50} value of component LsIII assayed by intramuscular injection in mice was 1.24μg/g body wt. It is about 8.5 times less toxic than erabutoxins a, b and c. The toxic symptoms produced in mice by component LsIII were similar to those of erabutoxins and other post-synaptically acting snake toxins. It induced flaccid paralysis of the extremities and the mice died from respiratory failure, usually within 1h, with surviving animals recovering in a few hours.

Lethal activity in baby chicks

The baby chick was more susceptible to component LsIII, as well as to erabutoxins, than the mouse.

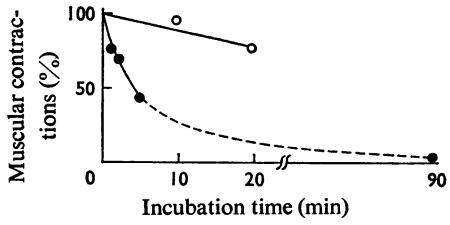

Fig. 2. *Effect of incubation with component LsIII on the acetylcholine contraction of frog rectus abdominis muscle*

Each muscle was placed in glucose–Ringer solution (5ml), through which O_2 was continuously bubbled. The contraction of the muscle by the addition of acetylcholine chloride (final concn. 0.5μg/ml) was measured before and after incubation with component LsIII, at final concentrations of (○) 0.83 and (●) 5μg/ml.

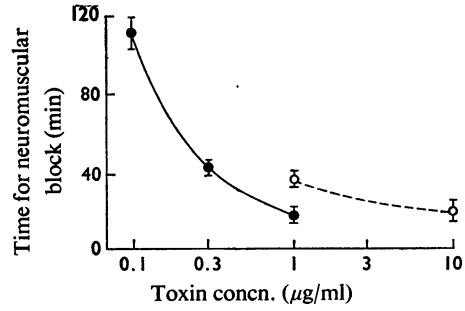

Fig. 4. *Relationship between concentration of neurotoxins and time for complete neuromuscular block of the chick biventer cervicis muscle preparation*

○, Component LsIII; ●, erabutoxin b. The vertical bars denote S.E. for five experiments. For details see the text.

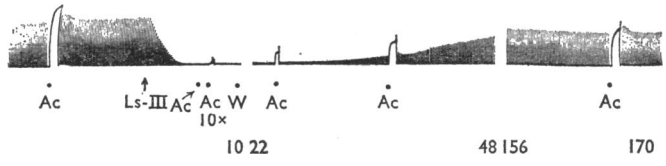

Fig. 3. *Effect of component LsIII on chick biventer cervicis nerve–muscle preparation*

Indirect supramaximal stimulation (0.1Hz, 0.5ms) was used. Electrical stimulation was interrupted during testing of acetylcholine response (Ac). The dose of acetylcholine was 20μg/ml, except at 'Ac 10x' which was 200μg/ml. At the arrow, the component LsIII was added (final concn. 50μg/ml). At point W and then every 10min, the preparation was washed with Krebs solution. Note that the acetylcholine response recovered earlier than the response to indirect stimulation.

The LD_{50} determined by intramuscular injection in baby chicks was 0.45μg/g body wt. for component LsIII and 0.043μg/g body wt. for erabutoxin b. Although the toxic symptoms produced in chicks by these materials were similar to those in mice, flaccid paralysis lasted much longer and in general, respiratory failure occurred more slowly in chicks than in mice.

Action on isolated rectus abdominis muscle of the frog

Component LsIII (5μg/ml) inhibited the contraction by acetylcholine (0.5μg/ml) of a frog rectus abdominis muscle by 50% 5min after addition to the medium. Erabutoxins give the same inhibition at 0.4μg/ml. Component LsIII did not affect the contraction of the muscle by KCl. At lower concentration (0.83μg/ml), component LsIII acted more slowly on the muscle, as shown in Fig. 2. The contraction by acetylcholine of the muscle, which was treated with component LsIII (5μg/ml) for 90min, recovered to 50% of the original value, when the muscle was washed (6×5ml) with glucose–Ringer solution (Tamiya & Arai, 1966) during 60min.

Action on the chick biventer cervicis muscle preparation

Unlike cardiotoxin (Lee *et al.*, 1968), component LsIII did not produce any contraction at a concentration as high as 50μg/ml. The twitch response of the muscle to indirect stimulation was blocked within 5min and the response to acetylcholine (20μg/ml) was also completely abolished (Fig. 3).

On repeated washing both the twitch response to indirect stimulation and the acetylcholine response recovered. By contrast, the neuromuscular block produced by erabutoxin b in this muscle preparation is irreversible (Lee *et al.*, 1972). The relation between the concentration of component LsIII and the time for complete neuromuscular block in this preparation is compared with that for erabutoxin b in Fig. 4.

Action on the rat phrenic-nerve–diaphragm preparation

The rat phrenic-nerve–diaphragm preparation was more resistant to component LsIII than was the chick biventer cervicis muscle preparation. At a concentration of 3μg/ml no complete neuromuscular block was observed even at 300min after application of the toxin. With 10μg of component LsIII/ml (Fig. 5),

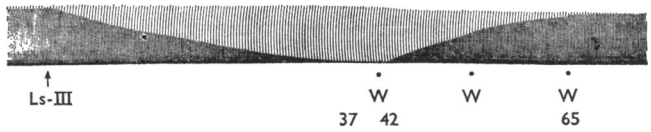

Fig. 5. *Effect of component LsIII on rat phrenic-nerve–diaphragm preparation*

Indirect (0.1ms) and direct (0.5ms) supramaximal stimulations were applied alternately every 10s. At the arrow, the component LsIII was added (final concn. 10μg/ml). At W, the preparation was washed with Tyrode's solution. Note the immediate recovery from the neuromuscular block.

the twitch height elicited by indirect stimulation decreased progressively and complete neuromuscular block took place at 53±8.1min (mean±S.E.M. of three experiments), whereas the twitch response to direct stimulation remained unaffected. After washing, the twitch response to indirect stimulation reappeared immediately and complete recovery took place within 30min. The rat diaphragm preparation is about ten times more sensitive to erabutoxin b than to component LsIII, and the neuromuscular block by erabutoxin b in this preparation has been found to be slowly reversible (Lee *et al.*, 1972).

Discussion

Nearly 40 'neurotoxins' and 'cardiotoxins' have been isolated from the venom of *Hydrophiidae* and *Elapidae* snakes and sequenced (Maeda & Tamiya, 1974). All the neurotoxins show about the same lethal activity (0.07–0.15μg/g body wt. in mice). These neurotoxins are classified into two groups, namely short-chain and long-chain toxins (Strydom, 1973). The former consist of 60–62 amino acid residues with four disulphide bridges, and the latter consist of 71–74 residues with five disulphide bridges. The cardiotoxin group includes cytotoxins and direct lytic factor and they are less lethal to animals than are neurotoxins (Lee, 1972). The cardiotoxins also consist of 60–62 amino acid residues with four disulphide bridges (Lee, 1972).

The sea-snake venom component LsIII reported here consists of 66 amino acid residues with five disulphide bridges (Maeda & Tamiya, 1974). The toxic symptoms produced by component LsIII in mice as well as in baby chicks were similar to those by erabutoxins, but component LsIII was about eight to ten times less toxic than the latter. The acetylcholine contraction of the frog rectus abdominis muscle was inhibited by component LsIII, whereas the KCl contraction of the muscle remained unaffected. In the chick biventer cervicis muscle, the acetylcholine contraction and the twitch response to indirect stimulation were also abolished by component LsIII. Similarly in the rat phrenic-nerve–diaphragm preparation, the twitch response to indirect stimulation was abolished by component LsIII, whereas that to direct stimulation remained unaffected. Unlike cardiotoxin, component LsIII did not produce contraction of skeletal muscle even at a high concentration. All of these findings indicate that component LsIII is a post-synaptically acting neurotoxin and not a cardiotoxin. The observation that a higher concentration of component LsIII was required and that component LsIII was more easily washed off as compared with other neurotoxins may suggest its lower affinity to acetylcholine-receptor sites.

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