Properties and Biosynthesis of a Neurotoxic Protein of the Venoms of Sea Snakes *Laticauda laticaudata* and *Laticauda colubrina*

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1. A neurotoxic protein similar to erabutoxins a and b of *Laticauda semifasciata* was isolated in crystalline form from the venoms of *Laticauda laticaudata* and *Laticauda colubrina*. The name 'laticotoxin a' is proposed. 2. Laticotoxin a is homogeneous by CM-cellulose column chromatography, disc electrophoresis and ultracentrifugation and by terminal amino acid analyses. 3. Laticotoxin a consists of 62 amino acid residues. The molecular weight by ultracentrifuging is 6520. 4. The minimal 50% lethal dose of laticotoxin a by intramuscular injection to mice is 0.13 $\mu\text{g./g.}$ body wt. The toxin attacks the postsynaptic membrane, competing with acetylcholine. 5. Radioactive amino acids are incorporated into laticotoxin *in vivo*. The incorporation is inhibited by puromycin, suggesting that the biosynthesis of the toxin follows the mechanism of protein biosynthesis, although the toxin molecule is rather small as a protein.

In a previous paper (Tamiya & Arai, 1966) the isolation and properties of neurotoxic proteins from the venom of a sea snake *Laticauda semifasciata* were described. They were named erabutoxins a and b. The present paper reports the isolation and properties of a very similar toxin from the venoms of closely related sea snakes *Laticauda laticaudata* and *Laticauda colubrina*. The name 'laticotoxin a' is proposed. The neurotoxicity of the venoms of these snakes has already been described (Tu, 1963).

It is also suggested in this paper that the biosynthesis of the laticotoxin a follows the mechanism described for the synthesis of any protein, because the biosynthesis is inhibited by puromycin (Yarmolinsky & de la Haba, 1959). It is generally believed that the polypeptide chains of proteins are synthesized on ribosomes through the participation of messenger RNA, but the smallest size of polypeptides produced by a ribosomal system is not known, nor is it known whether such a limit exists. Laticotoxin a, which consists of 62 amino acid residues, is one of the smallest proteins produced by the puromycin-sensitive ribosomal system.

MATERIALS AND METHODS

Sea snakes. Sea snakes *Laticauda laticaudata* and *Laticauda colubrina* were captured near the Okinawa Islands. The specimens used for the biosynthesis of the toxin were kept alive in the laboratory for 4 months in a water bath at 25°.

Crude dried venom. Snakes were anaesthetized with chloroform vapour. The venom ducts from the parotid

glands to the front upper teeth were stopped by Kocher's forceps. The glands of *L. laticaudata* were taken out and the contents squeezed on to glass and dried over NaOH pellets at -20°. The colourless dried powder thus obtained was further dried *in vacuo* for 8 hr. at room temperature. About 100 mg. of crude dried venom was obtained from ten individuals of *L. laticaudata*. The preparation was stored at -20° and had an LD₅₀ value of 0.17 $\mu\text{g./g.}$ body wt. of the mouse by intramuscular injection in the hind legs.

Two glands of a single specimen of *L. colubrina* were taken out and freeze-dried. The dried glands weighed 55.5 mg.

Radioactivity measurements. Radioactivity measurements were carried out with an Aloka low-background windowless gas-flow counter (type LBC-20; Nihonmusen Co., Tokyo, Japan).

Labelled amino acid mixture. Uniformly ¹⁴C-labelled amino acid mixture (*Chlorella* hydrolysate, 4.2 mc/mg. atom of C) was obtained from the Daiichi Chemical Co., Tokyo, Japan.

RESULTS

Isolation and properties of laticotoxin a

CM-cellulose column chromatography. A 25 mg. sample of crude dried venom of *L. laticaudata* was extracted with 0.01 M-sodium-potassium phosphate buffer, pH 6.4 (5 ml.), containing sodium chloride (0.01 M). The supernatant obtained by centrifuging (3000 g for 10 min.) was applied to a CM-cellulose column (0.9 cm. $\times$ 17 cm.) that had been equilibrated with the buffer. The sodium chloride concentration of the eluent was raised stepwise from 0.01 M to 0.02 M, to 0.04 M, to 0.1 M and to 0.5 M. The elution pattern of the proteins (*E* at 280 nm.) and that of

Fig. 1. CM-cellulose column chromatography of *Laticauda laticaudata* venom. ●, Protein content of the eluate determined by $E_{280}^{1\text{cm}}$; ○, toxicity of the eluate estimated by intramuscular injection into mice (Tamiya & Arai, 1966) and expressed in terms of the body weight of mice of which 50% can be killed by 1 ml. of the fractions. The largest peak corresponds to laticotoxin a. The points at which the molarity of the NaCl was changed are indicated by the arrows.

Table 1. Purification of laticotoxin a from venoms of *Laticauda laticaudata* and *Laticauda colubrina*

	Recovery (%)		LD ₅₀ (μg./g. body wt. of mouse)
	Protein	Toxicity	
Crude venom extracts (<i>L. laticaudata</i>)	100	100	0.17
Peak 1	18	0	
Peak 2 (laticotoxin a)	72	100	0.13
Recrystallized laticotoxin a (<i>L. laticaudata</i>)			0.13
Crude gland extracts (<i>L. colubrina</i>)	100	100	0.4
Peak 1	16.6	0	
Peak 2 (laticotoxin a)	38.2	100	0.13
Freeze-dried laticotoxin a (<i>L. colubrina</i>)			0.13

the toxicity are shown in Fig. 1. The toxic substance contained in the main peak was named 'laticotoxin a'.

Laticotoxin a was quantitatively recovered as a single peak on rechromatography on the same column. The purification is summarized in Table 1.

Two glands of *L. colubrina* were cut into pieces and extracted with 0.01 M-sodium-potassium phosphate buffer, pH 6.4 (5 ml.), containing sodium chloride (0.01 M). The supernatant was decanted and the residue re-extracted with the buffer (2 ml.). The combined supernatant was centrifuged (3000g for 15 min.) and applied to a CM-cellulose column (0.85 cm. × 19 cm.) that had been equilibrated with the same buffer. The sodium chloride concentration of the eluent was raised stepwise and essentially the same elution pattern as that of Fig. 1 was obtained. The purification is summarized in Table 1.

Fig. 2. Crystals of laticotoxin a from *Laticauda laticaudata* (× 500).

Fig. 3. Crystals of laticotoxin a from *Laticauda colubrina* (× 500).

The u.v.-absorption curves of both toxic peaks were those of typical proteins.

Crystallization of laticotoxin a. The laticotoxin a fractions from 75 mg. of crude dried *L. laticaudata* venom were freeze-dried and the residue was dissolved in 0.15 M-sodium chloride solution (1 ml.). Saturated ammonium sulphate solution was added to make 60% saturation. The solution was left at 4° overnight. The needle crystals deposited were collected by centrifugation (1000g for 10 min.), dissolved in 0.15 M-sodium chloride solution (0.5 ml.) and recrystallized by the addition of saturated ammonium sulphate solution (Fig. 2).

The fractions of the toxic main peak (total 9.4 E_{280} units) of the CM-cellulose column chromatography of *L. colubrina* venom were freeze-dried. The residue was dissolved in water (5 ml.) and the solution dialysed against water (100 ml. and 150 ml.) and freeze-dried. About 9 mg. of colourless powder was obtained. A 3 mg. sample of the powder was dissolved in a drop of water and saturated ammonium sulphate solution was added until the solution became faintly turbid. After the solution

Fig. 4. Schematic disc electrophoretograms of sea-snake toxins at pH 4.3. E-a, Erabutoxin a; E-b, erabutoxin b; L-a, laticotoxin a.

Fig. 5. Ultracentrifugation of laticotoxin a. Laticotoxin a was dissolved in 0.05M-phosphate buffer, pH 7.00, containing NaCl (0.1M) ($E_{280}^{1\text{cm}}$ 6.9) and centrifuged at 34500g at 19°. The picture was taken 6 min. after constant velocity was reached.

was kept at room temperature overnight, crystals were deposited (Fig. 3).

Disc electrophoresis of laticotoxin a. The crystals of laticotoxin a (*L. laticaudata*) were separated from the mother liquor and dissolved in water ($E_{280}^{1\text{cm}}$ 1.0). The freeze-dried toxin powder of *L. colubrina* (see above) was dissolved in water (1mg./ml.). One-tenth of each solution was subjected to disc electrophoresis at pH 4.3 as described by Tamiya & Arai (1966) for erabutoxins. The results are schematically shown in Fig. 4. The electrophoretograms of erabutoxins a and b are also shown for comparison. Both toxins from *L. laticaudata* and *L. colubrina* moved towards the cathode as single bands. The mobility of both toxins was the same on mixed electrophoresis.

Ultracentrifuging of laticotoxin a. Laticotoxin a fractions (*L. laticaudata*) from CM-cellulose column

Table 2. Amino acid composition of sea-snake toxins

Toxin	...	Laticotoxin a		Erabu-	Erabu-
		<i>L. laticaudata</i>	<i>L. colubrina</i>	toxin a*	toxin b*
Species	...			<i>L. semi-fasciata</i>	<i>L. semi-fasciata</i>
Asp + Asn		8.75 (9)	8.6 (9)	5.08 (5)	4.15 (4)
Thr		3.95 (4)	4 (4)	4.99 (5)	4.95 (5)
Ser		5.9 (6)	6 (6)	7.8† (8)	8† (8)
Glu + Gln		7.3 (7)	7.0 (7)	8.17 (8)	8.08 (8)
Pro		5.3 (5)	5.3 (5)	4.17 (4)	4.15 (4)
Gly		5.0 (5)	5.2 (5)	5.09 (5)	4.89 (5)
Ala		0	0	0	0
Cys		7.6 (8)	7.97 (8)	8.00 (8)	7.94 (8)
Val		1.0 (1)	0.9 (1)	2.00 (2)	1.99 (2)
Met		0	0	0	0
Ile		2.25 (2)	2.1 (2)	3.65 (4)	3.81 (4)
Leu		1.1 (1)	1.0 (1)	1.06 (1)	1.13 (1)
Tyr		1.25 (1)	0.9 (1)	0.84 (1)	0.92 (1)
Phe		1.1 (1)	1.0 (1)	2.09 (2)	1.99 (2)
Lys		4.1 (4)	3.5 (4)	3.92 (4)	4.01 (4)
His		2.0 (2)	2.1 (2)	0.91 (1)	1.99 (2)
Arg		5.0 (5)	5.48 (5)	2.98 (3)	2.97 (3)
Trp		1 (1)	1 (1)	1.31 (1)	1.40 (1)
Total		62	62	62	62
Minimal mol.wt.		6970	6970	6850	6870
Mol.wt. by centrifuging		6520		7430	7430

* Data from Tamiya & Arai (1966).

† One more serine was found by 12hr. hydrolysis as compared with the previous data.

chromatography were freeze-dried and the residue was dissolved in 0.1M-acetic acid (4ml.). The solution was passed through a Sephadex G-25 column (2.4 cm. × 30 cm.) and the protein-containing fractions detected by $E_{280}^{1\text{cm}}$ were freeze-dried. The salt-free laticotoxin a thus obtained was dissolved in 0.05M-sodium-potassium phosphate buffer, pH 7.0, containing sodium chloride (0.1M) and dialysed against the same buffer. A solution with $E_{280}^{1\text{cm}}$ 6.9 was obtained. The solution was subjected to approach-to-equilibrium sedimentation of Archibald (Schachman, 1959) in a cell described by Yphantis (1960). The partial specific volume was calculated from the amino acid composition (see below) to be 0.71 (Cohn & Edsall, 1943). Laticotoxin a is ultracentrifugally monodisperse (Fig. 5). A molecular weight of 6520 was obtained.

Amino acid composition and N- and C-terminal amino acids of laticotoxin a. Salt-free freeze-dried toxins of *L. laticaudata* and *L. colubrina* (each 0.5mg.) were hydrolysed with 6M-hydrochloric acid (0.5ml.) in sealed glass tubes *in vacuo* at 110° for 11, 22, 44 and 66 hr. The amino acids in the

hydrolysates were analysed by an automatic amino acid analyser (type JLC-5AH; Japan Electron Optics Laboratory Co., Tokyo, Japan). The results are shown in Table 2. The tryptophan content of the toxins was determined by u.v. absorption as described by Goodwin & Morton (1946). Both toxins consisted of 62 amino acid residues and had the same composition. The amino acid compositions of erabutoxins a and b are also shown for comparison. The minimal molecular weight calculated from the amino acid composition agreed well with the molecular weight obtained by centrifuging (see above).

Laticotoxin a from *L. laticaudata* (2.2 mg.) or *L. colubrina* (0.9 mg.) was dissolved in water (0.2 ml.) and dinitrophenylated with 1-fluoro-2,4-dinitrobenzene (20 mg.) in ethanol (0.4 ml.) as described by Sanger (1949). The dried dinitrophenylated toxin was suspended in water (0.1 ml.) containing tritium oxide (60 mc), and pyridine (0.2 ml.) and acetic anhydride (0.06 ml.) were added to label the C-terminal amino acid (Matsuo, Fujimoto & Tatsuno, 1966). The mixture was kept at room temperature for 8 hr., diluted with water (3 ml.) and centrifuged (3000 g for 10 min.). The red precipitate was washed twice with water (each 3 ml.) and dried *in vacuo*. Dinitrophenylated and tritiated toxin thus obtained was hydrolysed *in vacuo* in a sealed glass tube with 6 M-hydrochloric acid (0.5 ml.) for 24 hr. at 110°. The hydrolysate was dried *in vacuo* over sodium hydroxide pellets. DNP-arginine in the hydrolysate was detected by paper chromatography and paper electrophoresis. The paper chromatogram (butan-1-ol-acetic acid-water, 4:1:2, by vol.) of the hydrolysate was cut at every 5 mm. and the radioactivity of the paper pieces counted by a windowless counter. Radioactive aspartic acid was detected from both toxins. The results show that laticotoxin a from both *L. laticaudata* and *L. colubrina* has arginine and asparagine or aspartic acid (Matsuo *et al.* 1966; Holcomb, James & Ward, 1968) at its N- and C-termini respectively.

Toxicity of laticotoxin a. The lethal dose of the laticotoxin a from both *L. laticaudata* and *L. colubrina* was determined by injection into the hind-leg muscle of the mouse (average body weight 15 g.) as a solution in 0.15 M-sodium chloride. The LD₅₀ values of laticotoxin a from both species were 0.13 µg./g. body wt. of the mouse. A solution of laticotoxin a ($E_{280}^{1\text{cm}}$ 0.12) in 0.15 M-sodium chloride solution lost less than 30% of its activity in 15 min. at 100°.

Mode of action of laticotoxin a. An isolated frog rectus abdominis muscle (*Rana nigromaculata*) was placed in 5 ml. of glucose-Ringer solution (6.43 g. of sodium chloride, 0.30 g. of potassium chloride, 0.17 g. of calcium chloride, 0.10 g. of sodium hydrogen carbonate and 0.71 g. of glucose in 1000 ml.

of water) that was continuously bubbled with air. The contraction of the muscle was recorded by a strain-gauge transducer as described by Tamiya & Arai (1966). Laticotoxin a from *L. laticaudata* or *L. colubrina* was added to the medium at the final concentration of 0.06 µg./ml. and after 20 min. acetylcholine chloride (final concn. 0.5 µg./ml.) was added. The contraction of the muscle was completely abolished whereas the muscles in control experiments without the toxin contracted on the addition of acetylcholine. When potassium chloride was added (final concn. 0.014 M) instead of acetylcholine chloride, the muscle with the toxin contracted more strongly than the muscle without the toxin. The results suggest that laticotoxin a acts at the postsynaptic membrane, competing with acetylcholine. The mode of action of laticotoxin a is the same as that of erabutoxins.

Biosynthesis of laticotoxin a

Incorporation of labelled amino acids into laticotoxin a in vivo. A snake (*L. laticaudata*) was injected in the venom glands with 5 µC of ¹⁴C-labelled amino acid mixture. After 3 hr. the snake was killed by decapitation and the venom glands were taken out immediately. The glands were homogenized in 0.01 M-sodium-potassium phosphate buffer, pH 6.4 (5 ml.), containing sodium chloride (0.01 M). The homogenate was centrifuged (3000 g for 10 min.) and the supernatant passed through a Sephadex G-25 column (2.7 cm. × 40 cm.) equilibrated with the same buffer. Protein was separated from the low-molecular-weight substances by the procedure. Total radioactivity associated with the protein

Fig. 6. CM-cellulose column chromatography of protein fraction from Sephadex G-25. The protein fraction was loaded on a CM-cellulose column (0.9 cm. × 20 cm.) that had been equilibrated with 0.01 M-phosphate buffer, pH 6.4, containing NaCl (0.01 M). Elution was carried out by a stepwise raising of NaCl concentration in the buffer. The largest peak corresponds to laticotoxin a. ●, E_{280} ; ○, radioactivity.

Fig. 7. Paper electrophoresis of tryptic peptides from oxidized laticotoxin a. Experimental details are given in the text. Arginine was run by the side of the sample as standard.

Table 3. Total radioactivity of tryptic peptides from oxidized laticotoxin a

Counts are given above background (3 c.p.m.).

Peptide	Radioactivity (c.p.m.)
I	28
II	102
III	37
IV	106
V	27
VI	6

fraction was 3880 c.p.m. The protein fraction was submitted to CM-cellulose column chromatography as described above. The elution profile of the proteins (E_{280}^{1cm}) and radioactivity is given in Fig. 6. The total radioactivity eluted in coincidence with laticotoxin a was 2750 c.p.m., which accounts for 70% of the radioactivity applied to the column.

The labelled laticotoxin a fractions (total 40 ml.) were desalted by means of Diaflo apparatus (Amicon Corp., Lexington, Mass., U.S.A.) with UM-05 membrane; 20 ml. of water was used for desalting. The desalted laticotoxin a was dissolved in water (2 ml.) and freeze-dried. A 1 mg. sample of the labelled laticotoxin a was oxidized with performic acid as described by Thompson (1954). The oxidized toxin was dissolved in 0.05 M-tris-hydrochloric acid buffer, pH 7.9 (0.1 ml.), and digested overnight at 25° with 10 µg. of trypsin (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). One half of the mixture was transferred to filter paper (Toyo no. 51; Toyoroshi Co., Tokyo, Japan) and subjected to electrophoresis at 60 v/cm. for 60 min. in the presence of a buffer (acetic acid-pyridine-water, 10:1:289, by vol.) at pH 3.7. The tryptic peptides separated were revealed by ninhydrin spray. Each spot was cut out and its

radioactivity determined. All six spots, designated I-VI, were found to be radioactive, although the activity of spot VI, which seemed to be free arginine, probably from the N-terminus, was rather weak (Table 3).

The results show that ^{14}C -labelled amino acids are incorporated into the laticotoxin a molecule *in vivo* in *L. laticaudata*.

Inhibition of amino acid incorporation by puromycin. A snake (*L. laticaudata*) was injected in the venom glands with 5 µc of ^{14}C -labelled amino acid mixture together with puromycin (100 µg.). After 3 hr. the snake was killed and laticotoxin a was isolated from the glands as described above. Very little radioactivity (42 c.p.m./mg.) was detected in laticotoxin a isolated from the snake, but a considerable radioactivity (706 c.p.m./mg.) was incorporated into the toxin in a control experiment without puromycin. The amino acid incorporation was inhibited to the extent of 94% by puromycin.

DISCUSSION

A neurotoxic protein was isolated from the venoms of sea snakes *L. laticaudata* and *L. colubrina* in a crystalline form. The amino acid composition and other properties of the toxin preparations from both species were the same. It was concluded that these two species produce the same toxin and the toxin was named laticotoxin a.

The laticotoxin a is homogeneous by CM-cellulose column chromatography, disc electrophoresis and ultracentrifuging and by N- and C-terminal amino acid analyses.

The laticotoxin a is very similar to erabutoxins a and b isolated by Tamiya & Arai (1966) from *Laticauda semifasciata* in molecular size (62 amino acid residues), N- and C-terminal amino acids (arginine and asparagine or aspartic acid respectively; the C-terminal amino acids of erabutoxins were obtained by S. Sato & N. Tamiya, unpublished work), lethal dose and the mode of action. The differences between erabutoxins and laticotoxin a are the replacement of nine amino acid residues out of total 62 residues and the higher positive charge that results in the higher electrophoretic mobility and stronger adsorption on CM-cellulose.

It is noteworthy that 100% of the toxicity and 70% of the protein in the venom are ascribed to a single crystallizable neurotoxic protein, laticotoxin a. About 70% of the radioactivity incorporated into the protein fractions of the venom gland extracts was found in laticotoxin a. The venom gland is thus an organ differentiated for the specific synthesis of the toxin.

The coincidence of the elution patterns of laticotoxin a and the radioactivity and the incorporation of labelled amino acids into the tryptic

peptides of the toxin are good criteria for new synthesis of the toxin in the glands.

The fact that the biosynthesis of laticotoxin a is inhibited by puromycin, which is known to block protein synthesis at a very early stage (Yarmolinsky & de la Haba, 1959), suggests that the biosynthesis of the toxin follows the pattern of protein synthesis. This provides a reason for laticotoxin a to be called a protein, although the molecule is rather small. The toxin is one of the smallest proteins synthesized by a ribosomal system, being smaller than proinsulin (Steiner, Cunningham, Spiegelman & Aten, 1967).

A preliminary experiment was carried out on the turnover of RNA in venom glands with inorganic [^{32}P]phosphate or [^{14}C]orotic acid. It was found that the glands had relatively low activity of RNA synthesis. Moreover, all RNA fractions were labelled in a rather homogeneous way, and no RNA fraction with a high metabolic turnover rate could be demonstrated. The messenger RNA for laticotoxin a thus seems to be relatively stable, as in most of the cases of differentiated cells for a specific protein synthesis from animal sources, such as the reticulocyte (Marks, Willson, Kruh & Gros, 1962).

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