Isolation, Properties and Amino Acid Sequences of Three Neurotoxins from the Venom of a Sea Snake, *Aipysurus laevis*

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Aipysurus laevis venom was chromatographed on CM-cellulose and Bio-Rex 70 columns. Three neurotoxic components, toxins Aipysurus laevis a, b and c, were isolated. The toxins a, b and c corresponded to 22, 33 and 21% respectively of the proteins in the original venom, and accounted for almost all the lethal activity of the venom. The three toxins a, b and c were monodisperse on disc electrophoresis at pH4; toxins a and b moved at the same velocity and c a little faster. They were monodisperse also on sodium dodecyl sulphate–polyacrylamide-disc-gel electrophoresis, giving a molecular weight of 7600. The molecular weight of toxin b estimated by gel filtration was 7000. The amino acid sequence analyses of these toxins revealed that they consisted of 60 amino acid residues and that Aipysurus laevis b was [25-methionine, 28-arginine] Aipysurus laevis a. Aipysurus laevis c was [28-lysine]Aipysurus laevis a, the tryptic peptide sequence relying on homology. The LD₅₀ values of these toxins for 20g mice were 0.076 µg/g body wt. They inhibited the acetylcholine-induced contracture but did not affect the KCl-induced contracture of the isolated muscle. Detailed evidence for the amino acid sequences of the proteins has been deposited as Supplementary Publication SUP 50057 (11 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1976), 153, 5.

Aipysurus laevis is one of the common sea snakes in the northern and western waters of Australia. During the sea-snake expedition on the research vessel 'Alpha Helix' of Scripps Institution of Oceanography from December 1972 to January 1973, 64 *Aipysurus laevis* snakes were collected.

By successive chromatography of the *Aipysurus laevis* venom on CM-cellulose and Bio-Rex 70 columns three strongly neurotoxic components named toxins Aipysurus laevis a, b and c, were obtained.

The present paper describes the isolation, some properties and amino acid sequences of these three toxins.

Materials and Methods

Sea snakes

Milking and the toxicity of the venom from the live sea snakes, *Aipysurus laevis*, were described by Tamiya & Puffer (1974). The venom was used fresh on the research vessel or diluted with water, freeze-dried and brought back to the laboratory.

Proteinases

Trypsin (twice crystallized, salt-free), and α -chymotrypsin (three-times crystallized) were the products of Worthington Biochemical Corp., Free-

hold, N.J., U.S.A. Carboxypeptidase A (treated with di-isopropyl phosphorofluoridate; from bovine pancreas) and leucine aminopeptidase (from hog kidney) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Thermolysin (three-times crystallized) was purchased from Seikagaku Kogyo Co., Tokyo, Japan.

Reagents

Phenyl isothiocyanate was the 'Sequenal grade' of Wako Pure Chemical Industries, Osaka, Japan. Dimethylallylamine was synthesized from allyl chloride and dimethylamide (Ahlroth, 1965). Mono-iodoacetic acid was recrystallized from chloroform/light petroleum (b.p. 30–70°C). 2-Mercaptoethanol was redistilled under reduced pressure before use. Benzene and ethyl acetate were redistilled and other solvents and reagents were used without further purification.

Column chromatography

The elution of the proteins from the columns was followed by measuring the extinction at 280 nm continuously by using a Beckman DB spectrophotometer (Beckman Instruments, Fullerton, Calif., U.S.A.) or by a Toyo Uvicon 540 M instrument (Toyo Scientific Instruments Co., Tokyo, Japan). CM-cellulose (Brown: capacity 0.70 mequiv./g) and

DEAE-cellulose (Brown: capacity 0.84 mequiv./g) were purchased from Seikagaku Kogyo Co., Tokyo, Japan.

Disc electrophoresis

The freeze-dried venom and its components were subjected to disc electrophoresis in 15% polyacrylamide gels at pH 4.0 as described by Reisfeld *et al.* (1962), with slight modifications described previously (Tamiya & Arai, 1966). Sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis was performed in 0.1% sodium dodecyl sulphate/10% acrylamide as described previously (Maeda *et al.*, 1974). Protein bands in the gels were detected by staining with Amido Black (1%, w/v, in 7%, v/v, acetic acid) for 15 min and de-staining in 7% (v/v) acetic acid overnight.

Amino acid analysis

Proteins or peptides (approx. 0.05 μ mol) were hydrolysed with 0.4 ml of 6M-HCl in sealed tubes *in vacuo* at 110°C, usually for 24 h. The hydrolysates were analysed with an automatic amino acid analyser (type JLC-5AH; Japan Electron Optics Laboratory Co., Tokyo, Japan). The tryptophan content of the toxins was determined by u.v. absorption as described by Goodwin & Morton (1946).

Toxicity measurement

The LD₅₀ values of the venom and toxins were determined by injection into the hind leg muscles of mice (body wt. approx. 20 g) as described previously (Tamiya & Arai, 1966).

Effects on isolated muscle preparations

The action of the neurotoxins on the isolated rectus abdominis muscle of the frog *Rana nigromaculata* was studied as described previously (Tamiya & Arai, 1966) with SB-1T force-displacement transducer and multipurpose recorder RH-20 (Nihon Koden Co., Tokyo, Japan).

Reduction and S-carboxymethylation of the toxins

Toxins a, b and c were reduced and S-carboxymethylated as described by Crestfield *et al.* (1963). Urea and excess of reagents were removed by gel filtration on a column (1.3 cm $\times$ 65 cm) of Sephadex G-25 (fine grade) in 0.1 M-acetic acid.

'Fingerprint mapping' of tryptic digests of reduced and S-carboxymethylated toxins

Each reduced and S-carboxymethylated toxin (approx. 2 mg) was dissolved in 0.05 M-NH₄HCO₃ buffer (0.5 ml), pH 7.8, and digested with trypsin at an enzyme/substrate ratio of 1:50 (w/w). After incubation for 16 h at 37°C the digests were freeze-dried, redissolved in water (0.5 ml) and freeze-dried again. About 300 μ g of the freeze-dried material was dissolved in water (20 μ l) and applied on a sheet

(40 cm $\times$ 40 cm) of no. 50 filter paper (Toyo Roshi Co., Tokyo, Japan) and subjected to paper electrophoresis in pyridine/acetic acid/water (8:5.5:986.5, by vol.), pH 4.8, at 40 V/cm for 75 min, followed by paper chromatography to the second dimension with butan-1-ol/acetic acid/water (4:1:2, by vol.) for 16 h by the ascending method. The paper electrophoresis was carried out in a *n*-hexane bath chilled at 4°C by a dichlorodifluoromethane compressor system (Electrophoresis apparatus type MS-PA, Fuji-Riken Co., Tokyo, Japan). Peptides were located on the paper by spraying with 0.2% ninhydrin in acetone, Ehrlich reagent (Smith, 1953), Pauli reagent (Mann & Leone, 1953) and Sakaguchi reagent (Jepson & Smith, 1953).

Enzymic digestions

Digestion of the reduced and S-carboxymethylated toxins with trypsin was performed in 0.05 M-Tris/HCl buffer, pH 8.0, for 16 h at 37°C at a substrate concentration of about 5 mg/ml. The enzyme/substrate ratio was 1:100 (w/w).

The reduced and S-carboxymethylated toxins a and b (1.5 μ mol each) were digested with α -chymotrypsin in 0.05 M-ammonium acetate buffer, pH 7.3 (1 ml), for 40 min at 37°C. The enzyme/substrate ratio was 1:60 (w/w). The digestion was terminated by the addition of 1 M-acetic acid (0.5 ml) and the mixture freeze-dried. The freeze-drying was repeated again after the addition of water (1 ml).

Citraconylation followed by tryptic digestion of reduced and S-carboxymethylated toxin b were carried out as described previously (Maeda & Tamiya, 1974).

The carboxypeptidase A or leucine aminopeptidase digestion was carried out in 0.05 M-Tris/HCl buffer, pH 8.0, containing 25 mM-MgCl₂, at 37°C. The substrate concentration was about 1 mg/ml. The enzyme/substrate ratio was 1:20–50 (w/w). The digests were directly subjected to amino acid analyses.

Separation of the digestion fragments

The digestion fragments were separated from each other by combinations of chromatography on columns of Sephadex G-25 (column size given in the Results section), Bio-Rad Aminex A-4 (see below) and DEAE-cellulose (1.2 cm $\times$ 14 cm), and high-voltage paper electrophoresis in pyridine/acetic acid/water (8:5.5:986.5, by vol.), pH 4.8, or in 0.1 M-triethylamine solution adjusted to pH 8.8 with acetic acid.

The Aminex A-4 column (0.9 cm $\times$ 55 cm), equipped with a water jacket, was maintained at 37°C and had been equilibrated with 0.1 M-pyridine/acetate buffer (8 ml of pyridine and 130 ml of acetic acid/1000 ml, pH 3.1). Stepwise elution was carried out with the same buffer, 0.5 M, pH 4.1 (4 ml of pyridine and 14 ml of acetic acid/200 ml), 1 M, pH 5.1 (16.1 ml and 14.5 ml/200 ml), 2.0 M, pH 5.6 (36.8 ml and 5 ml/200 ml), and 4 M (73.6 ml and 10 ml/200 ml). The volume of each

buffer used varied between 54 and 162 ml. Fractions (4.5 ml) were collected at a flow rate of 54 ml/h and 0.1 ml of each fraction was checked for ninhydrin reaction after alkaline hydrolysis (Hirs *et al.*, 1956).

Edman degradation

The amino acid sequences of the peptides and the reduced and *S*-carboxymethylated toxins were determined by the direct phenylthiohydantoin method of Iwanaga *et al.* (1969). The ethyl acetate-soluble phenylthiohydantoin derivatives were identified by t.l.c. In some cases the amino acid released was also identified subtractively by amino acid analysis of a portion of the peptide remaining after a round of degradation.

Fig. 1. CM-cellulose column chromatography of *Aipysurus laevis* venom

The crude venom (163.3 mg) from 1.23 ml of the venom was dissolved in 0.01 M-phosphate buffer, pH 6.4, and chromatographed on a CM-cellulose column (1.6 cm $\times$ 35 cm) which had been equilibrated with the same buffer. At arrow 1 the linear NaCl concentration gradient was applied, with 300 ml of 0.01 M-phosphate buffer, pH 6.4, in the mixing chamber and the same buffer containing 0.2 M-NaCl in the reservoir. At arrows 2, 3 and 4, the NaCl concentration in the eluting buffer was changed to 0.2, 0.4 and 1 M respectively. The flow rate was 150 ml/h. The protein fractions underlined, a and b (110 ml) and c (75 ml), were collected., NaCl concentration.

Results

Isolation of neurotoxins

The crude venom (1.23 ml) was freeze-dried (163.3 mg) and dissolved in 0.01 M-phosphate buffer (20 ml) made of KH_2PO_4 and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, pH 6.4, and applied to a CM-cellulose column (1.6 cm $\times$ 35 cm) equilibrated with the same buffer. The NaCl concentration in the eluent was raised linearly to 0.2 M. The elution pattern is shown in Fig. 1. The recovery of the protein and the toxicity to mice in the elution components are summarized in Table 1. The protein fractions with strong lethal activity were pooled separately, concentrated and desalted in a Diaflo apparatus with a UM-2 filter (Amicon Corp., Lexington, Mass., U.S.A.), and freeze-dried.

The first neurotoxic component in Fig. 1 gave a single band on disc electrophoresis with or without sodium dodecyl sulphate, but the analysis of the tryptic fragments of the reduced and *S*-carboxymethylated derivative suggested a co-elution of at least two components. Further fractionation was carried out on a Bio-Rex 70 (-400 mesh) column (1.2 cm $\times$ 18 cm) prepared as described by Karlsson *et al.* (1971). Two components, toxins *Aipysurus laevis* a and b, were obtained as shown in Fig. 2. The ratio of toxins a to b varied with preparations from 3:7 to 5:5 (w/w).

The component in the second toxic fraction of CM-cellulose chromatography (Fig. 1) was single and named toxin *Aipysurus laevis* c.

Disc electrophoresis of toxins *Aipysurus laevis* a, b and c

The electrophoresis was performed at constant current of 3 mA per gel for 3 h. Toxins a, b and c from *Aipysurus laevis* all moved toward the cathode as single bands. At pH 4.0 toxin c moved a little farther (4.5 cm) than toxin a and b, whereas the latter two were inseparable from each other (4.0 cm). Under the same condition the distances of migration of erabutoxins a and b were 2.4 and 3.0 cm respectively.

Molecular-weight estimation

Molecular weights of toxins *Aipysurus laevis* a, b and c were estimated to be about 7600 from their

Table 1. Recovery of toxic components of *Aipysurus laevis* venom in CM-cellulose column chromatography

LD₅₀ was determined by intramuscular injection. M.U. (mouse unit) is the number of mice (body wt. 20 g) which can be killed (50% probability) by the fraction.

	Total absorbance [E $\times$ volume (ml)]	Dry wt. (mg)	LD ₅₀ (μ g/g)	M.U.
Venom (1.23 ml)	180 (100%)	163	0.12	68000
I	48.7 (27.1%)			
a, b	83.9 (46.6%)	72.6	0.067	54000
c	29.0 (16.1%)	21.6	0.067	19000
Recovery	161.6 (89.6%)			73000

Fig. 2. Separation of toxins a and b on Bio-Rex 70 column

The freeze-dried fraction a, b (Fig. 1, 15.7 mg) was dissolved in 0.02M-ammonium acetate buffer, pH7.5, and chromatographed on a Bio-Rex 70 (-400 mesh) column (1.2cm×18cm). The column was first equilibrated with 0.2M-ammonium acetate buffer, pH7.5, and before application of the sample, 20 ml of 0.02M buffer was added. At the arrow a linear gradient of buffer concentration from 0.05M (150 ml) to 0.2M (150 ml) was applied. The flow rate was 15 ml/h. The protein fractions underlined, a (10 ml) and b (15 ml), were collected.

Table 2. Amino acid compositions of toxins a, b and c

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

	Toxin a	Toxin b	Toxin c
Lysine	5.65 (6)	4.73 (5)	7.00 (7)
Histidine	0.84 (1)	0.89 (1)	1.02 (1)
Arginine	3.02 (3)	4.03 (4)	2.99 (3)
Aspartic acid	7.94 (8)	8.03 (8)	7.89 (8)
Threonine	6.77 (7)	6.88 (7)	6.67 (7)
Serine	2.91 (3)	2.91 (3)	2.86 (3)
Glutamic acid	7.68 (8)	7.03 (7)	6.94 (7)
Proline	2.83 (3)	2.91 (3)	2.67 (3)
Glycine	4.01 (4)	4.01 (4)	4.06 (4)
Alanine	1.02 (1)	1.01 (1)	1.10 (1)
½ Cystine*	7.64 (9)	8.25 (9)	7.22 (9)
Valine	1.37 (1)	1.25 (1)	1.09 (1)
Methionine	— (0)	1.02 (1)	— (0)
Isoleucine	1.92 (2)	1.88 (2)	1.88 (2)
Leucine	2.01 (2)	1.95 (2)	1.99 (2)
Tyrosine	0.66 (1)	0.74 (1)	0.77 (1)
Phenylalanine	— (0)	— (0)	— (0)
Tryptophan†	(1)	(1)	(1)
Total	60	60	60
Molecular weight			
Formula	6725	6757	6724
Gel filtration		7000	
Disc electrophoresis	7600	7600	7600

* Cysteine residues tend to give smaller values. The round number 9 was confirmed by the sequence study.

† Measured spectrophotometrically (Goodwin & Morton, 1946).

mobilities in sodium dodecyl sulphate-polyacrylamide-gel electrophoresis, by using pepsin (mol. wt. 35000), trypsin (mol.wt. 23500) and erabutoxin b (mol.wt. 6800) as standard proteins (Weber & Osborn, 1969). Toxin b was eluted from a Sephadex

G-50 (fine grade) column (1.1 cm×60 cm) at the same position as erabutoxin a (mol.wt. 6800).

Amino acid compositions

The results of amino acid analysis of 24h hydrolysates of each toxin are given in Table 2. The values were obtained from three to five duplicated analyses. No corrections were made.

All of the toxins are composed of 60 amino acid residues. Compared with toxin a, toxin b contains one more arginine and one more methionine residue and one less lysine and one less glutamic acid residue, and toxin c, one more lysine residue and one less glutamic acid residue. The true number of half-cystine residues of each toxin was obtained from the sequence study. Toxin b (2mg) treated with monoiodoacetic acid for 16h at 37°C in Tris/HCl buffer, pH8.3, containing 8M-urea (1.0ml) yielded 0.5 residue of carboxymethylcysteine/molecule on amino acid analysis. No carboxymethylation of toxin c (10mg) occurred under the same condition.

The molecular weights of toxins a, b and c, calculated from the amino acid compositions, are 6725, 6757 and 6724 respectively.

Toxicities and mode of action on isolated muscle

The LD₅₀ values of toxins a, b and c, determined from very steep dose-lethality curves obtained by injection into the hind leg muscles of mice, were all 0.076 µg/g body wt. The toxins are twice as toxic as erabutoxins a and b. Intramuscular injection of the toxins caused paralysis of hind legs, like other neurotoxins.

The contracture of the rectus abdominis muscle of a frog (*Rana nigromaculata*) by 0.5 µg/ml of acetylcholine in glucose/Ringer solution (Tamiya & Arai, 1966) was completely inhibited by toxin c and by a mixture of toxins a and b (the desalted and freeze-dried material from the first toxic fraction of CM-cellulose column chromatography, Fig. 1; final concentration 1 µg/ml) within 10 min. Three washings (each 5 ml) with glucose/Ringer solution did not decrease the inhibition. The contracture of the muscle induced by KCl (final concn. 0.05M) was not affected by the toxins. Fig. 3 shows the time-course of the contracture decrease after the toxins (final concn. 0.08 µg/ml) were added to the glucose/Ringer solution. The time required for 50% inhibition of the muscle contracture was 3 min for the mixture of toxins a and b or for toxin c, whereas that for erabutoxin a or b at the same concentration was 8 min.

Amino acid sequences

'Fingerprint maps' of tryptic digests. A comparison of 'fingerprint maps' of tryptic digests from reduced and S-carboxymethylated toxins a, b and c showed seven common peptides (Fig. 4). The peptide TIIb'

Fig. 3. Effect of incubation with toxins on the acetylcholine-induced contracture of frog rectus abdominis muscle

Each muscle was placed in glucose/Ringer solution (5 ml), through which O_2 was constantly bubbled. The contraction of the muscle by the addition of acetylcholine chloride (final concn. $0.5 \mu\text{g/ml}$) was measured before and after incubation with toxins (Tamiya & Arai, 1966). ●, Toxins *Aipysurus laevis* a and b mixture (see the text); ○, toxin *Aipysurus laevis* c; ■, erabutoxin b.

from toxins a and c seems to be an incomplete digestion product, consisting of peptides TIIb and TIIIc (lysine). The peptide TIIb' was not detected in the tryptic digestion mixture in 0.05M-Tris/HCl buffer, pH 8.0.

The peptides in spots TIV, TV and TVI, specific to toxins a, b and c respectively, were Ehrlich-positive. The naming of the spots was done from the mobilities on paper electrophoresis and paper chromatography of each peptide separated on columns (see below).

Separation of tryptic peptides. A mixture of toxins a and b (the desalted and freeze-dried material from the first toxic fraction of CM-cellulose column chromatography, Fig. 1; approx. $5 \mu\text{mol}$) was reduced and *S*-carboxymethylated and digested with trypsin as described in the Materials and Methods section. The tryptic peptide mixture was chromatographed on a Sephadex G-25 (fine grade) column ($1.2 \text{ cm} \times 270 \text{ cm}$) with 0.1 M-acetic acid (Fig. 5). Peptides TIV and TV, retarded on the column by an adsorption effect, were shown to be pure by paper electrophoresis at pH 4.8. Fraction TI was separated into peptides TIIa, TIIb and TIIc by preparative paper electrophoresis at pH 4.8. Fractions TII and TIII were applied on an Aminex column separately and chromatographed as described in the Materials and Methods section (elution patterns are given in Supplementary Publication SUP 50057). Fraction TIII was separated into five peptide fractions, TIIIa–TIIIe. Peptide TIIIe was further separated into peptides TIIIe-1 and TIIIe-2 by paper electrophoresis at pH 8.8. The main component of fraction TII was peptide TIIb.

The separation of the tryptic peptides of reduced

Fig. 4. 'Fingerprint maps' of tryptic digests of (a) reduced and *S*-carboxymethylated toxin a, (b) reduced and *S*-carboxymethylated toxin b, and (c) reduced and *S*-carboxymethylated toxin c

Details are given in the text. All the spots are ninhydrin-positive. The shaded spots are Pauli-positive. Peptides TIV, TV and TVI are Ehrlich-positive and TIIIc, TIIIe-1, TIIIe-2, TIV, TV and TVI are Sakaguchi-positive.

and *S*-carboxymethylated toxin c was made by the same method. Peptide TVI, retarded on the Sephadex G-25 (fine) column, was pure by paper electrophoresis at pH 4.8. The isolation, amino acid compositions and some properties of the tryptic peptides are given in the deposited data (Supplementary Publication SUP 50057). The amino acid compositions of the tryptic peptides agree with those shown in Fig. 6. The limits of error in this agreement are less than 0.1 residue for the peptides smaller than hexapeptides, and 0.5 residue for dodecapeptides, excluding the carboxymethylcysteine content which tends to give smaller values. The compositions of toxins a, b and c can be accounted for by peptides TIIIc and TIV, TV

Fig. 5. Gel filtration of tryptic digests of reduced and *S*-carboxymethylated toxin mixture a and b on Sephadex G-25 column

Elution was monitored by measuring E_{230} (—) and E_{280} (····). The flow rate was 30 ml/h. The peptide fractions TI (26 ml), TII (20 ml), TIII (26 ml), TIV (30 ml) and TV (36 ml) were collected.

and TIIIe-2, and TIIIC, TVI and TIIIe-2 respectively, in addition to seven common peptides, TIIa, TIIb, TIIb, TIIa, TIIb, TIIId and TIIIe-1.

Terminal amino acid sequence. The first 13 *N*-terminal amino acids of reduced and *S*-carboxymethylated toxin a were determined to be: Leu-Thr-CmCys-CmCys-Asn-Gln-Gln-Ser-Ser-Gln-Pro-Lys-Thr. The first 13 stages of Edman degradation of reduced and *S*-carboxymethylated toxin b and five stages of reduced and *S*-carboxymethylated toxin c gave identical sequences.

Carboxypeptidase A digestion at 37°C for 4 h yielded asparagine (1.25 mol), carboxymethylcysteine (0.22 mol) and glutamic acid (0.15 mol)/mol of reduced and *S*-carboxymethylated toxin a.

Amino acid sequences of tryptic peptides. Each peptide (0.5–2 μmol) was subjected to Edman degradation (Fig. 6). Asparagine and glutamine residues were confirmed by subjecting the leucine aminopeptidase digests of each peptide directly to amino acid analysis. Acid hydrolysate of peptide TIIb yielded 3.0 mol of aspartic acid/mol of peptide. After three amino acid residues had been removed from the *N*-terminus by the manual Edman method, the remaining peptide was digested with leucine aminopeptidase. Digestion for 2 h yielded carboxymethylcysteine (0.7), aspartic acid (1.0) and alanine (0.4), and digestion for 20 h released carboxymethylcysteine (1.8), aspartic acid (1.9), asparagine (0.6), serine (0.9), alanine (1.0) and tyrosine (0.9); the numbers in parentheses are mol of amino acid/mol of peptide. After seven steps of manual Edman degradation, digestion of the remaining peptide with leucine aminopeptidase for 20 h yielded carboxymethylcysteine (0.96), aspartic acid (0.18), asparagine (1.00), serine (0.80) and tyrosine (0.85). Basic amino acid

(lysine) was not analysed in the series of experiments. From these results the presence of aspartic acid at positions 4 and 7, and asparagine at position 8, in the peptide TIIb was deduced.

Amino acid analysis of the peptide TIIa showed that it contained two lysine residues, suggesting the presence of a Lys-Pro linkage which is resistant to tryptic digestion. Hydrolysis of the peptide with thermolysin (enzyme/substrate ratio, 1:50, w/w) at 37°C for 16 h yielded four peptides TIIa₁–TIIa₄, which were separated from each other by paper electrophoresis at pH 4.8. The amino acid compositions of these four peptides (given in Supplementary Publication SUP 50057) could account for that of peptide TIIa (Fig. 6). Six steps of Edman degradation of peptide TIIa accounted for the amino acid compositions of thermolytic peptides TIIa₁ and TIIa₂. Three steps of Edman degradation of tetrapeptide TIIa₃ and the existence of lysine residue in dipeptide TIIa₄ gave the amino acid sequence of the latter half of peptide TIIa.

Alignment of peptides. Tryptic peptide sequences were deduced as shown in Fig. 6 with the aid of the analysis of α -chymotryptic peptides from reduced and *S*-carboxymethylated toxins a and b, and tryptic peptides from citraconylated reduced and *S*-carboxymethylated toxin b.

Chymotryptic peptides from reduced and *S*-carboxymethylated toxin a were subjected to chromatography on a column (1.3 cm × 65 cm) of Sephadex G-25 (fine grade) with 0.1 M-acetic acid. The peptide-containing eluate, excluding the fraction containing peptide C-2, which was retarded on the column, was evaporated to dryness, applied on an Aminex A-4 column (0.9 cm × 55 cm) and chromatographed as described in the Materials and Methods section.

Two peptides, C-1 and C-3, were eluted by 0.1 M buffer and by 1.0 M buffer respectively. Three chymotryptic peptides, C-1, C-2' and C-3', from reduced and *S*-carboxymethylated toxin b were separated in the same way. Peptide C-1 from toxins a and b proved to be identical in chromatographic behaviour, amino acid composition, electrophoretic movement, and *N*-terminal two amino acids.

The tryptic digests of citraconylated reduced and *S*-carboxymethylated toxin b (approx. 30 mg) were desalted on a column of Sephadex G-25 (fine grade, 1.3 cm × 63 cm) in 0.1 M-acetic acid. The peptide in the first peak was evaporated to dryness and further subjected to chromatography on a DEAE-cellulose column (1.2 cm × 14 cm) in 0.01 M-phosphate buffer, pH 6.4, the NaCl concentration in the buffer solution being raised to 0.2 M linearly (100 ml in the mixing chamber). Two major peptides, A and B, were eluted at 95 and 120 ml respectively.

The second peptide peak which was eluted together with salts from the Sephadex G-25 column was separated into three components on an Aminex column under the conditions described in the Materials

Fig. 6. Amino acid sequences of *Aipysurus laevis* toxins a (a), b (b) and c (c)

Peptides are shown by bars with names. →, Amino acids detected by Edman degradation; ∩, amino acids detected by carboxypeptidase A digestion; ↓ and ↓ indicate the bonds cleaved by trypsin and α-chymotrypsin respectively; the longer arrows ↓ indicate the bonds cleaved in citraconylated derivatives. Key to one-letter notation for amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

and Methods section. The amino acid analysis of these peptides revealed them to be TIIIe-2, TIIIe-1 and TIIId.

Figs. 6(a), 6(b) and 6(c) show the whole amino acid sequences of the toxins a, b and c from *Aipysurus laevis* respectively. The results of amino acid analysis and the partial Edman degradation of the above-mentioned peptides (cf. Supplementary Publication SUP 50057) are explained by the sequences. The chymotryptic peptides permitted the placing of peptide TIIId and peptide TIV in toxin a, and peptide V and peptide TIIIe-2 in toxin b. The Edman degradation of peptide C-3 from toxin a gave the peptide sequence of TIV-TIIIe-1 and TIIId, and peptide C-3' from toxin b, the peptide sequence of TIIIe-2-TIIIe-1 and TIIId.

Discussion

The amino acid sequences of toxins a, b and c from *Aipysurus laevis* are shown in Fig. 6. Toxin b is [25-methionine,28-arginine]toxin a, and toxin c is [28-lysine]toxin a, the sequence of the tryptic peptide relying on homology to those of toxins a and b. Each amino acid substitution can be explained by a single base replacement in the triplet codes. The substitution does not affect the toxicity of the toxins. It is noteworthy that the positions 25 and 28, where the amino acid replacements occur, are quite near to the tryptophan residue at position 27, which is considered to be an indispensable residue for the toxicity from comparative sequence studies (cf. Table 5 of Maeda & Tamiya, 1974) and from chemical modification studies (Chang & Hayashi, 1969; Seto *et al.*, 1970; Chicheportiche *et al.*, 1972; Karlsson & Eaker, 1972; Chang & Yang, 1973).

As the toxins have nine half-cystine residues per molecule, at least one of them must be in a free-SH form. The quantitative carboxymethylation or 4-pyridylethylation (Fryklund *et al.*, 1972) of the free thiol group was unsuccessful even in the presence of 8M-urea. Although the position of the thiol group in *Aipysurus laevis* toxins has not been determined yet, that in *Enhydrina scistosa* toxins is tentatively located at position 3 by Fryklund *et al.* (1972).

With the ventral scales extending one-third of the width of the body, *Aipysurus* is classified by Smith (1926) as one of the members of the subfamily Laticaudinae, together with *Laticauda*, which has the wider ventral shields. On the other hand, in the subfamily Hydrophiinae, including *Enhydrina*, *Pelamis* and *Hydrophis*, ventrals at midbody are small or not differentiated. As far as the primary structures of the toxic components are concerned, *A. laevis* is more closely related to *E. scistosa* and *Hydrophis cyanocinctus* than to *Laticauda semifasciata* and *Laticauda laticaudata*, although the homology of all the toxins is clear (Fig. 7). Recently a proposal to separate

Laticauda from the family of Hydrophiidae was made (McDowell, 1967). The elucidation of the primary structures of the related toxins from various sea snakes will provide important information on the classification and evolution of sea snakes.

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