The Amino Acid Sequences of Erabutoxins, Neurotoxic Proteins of Sea-Snake (*Laticauda semifasciata*) Venom

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1. Erabutoxin b was reduced, *S*-carboxymethylated and hydrolysed with trypsin. Seven tryptic fragments were isolated by column chromatography and paper electrophoresis. Some of the fragments were further hydrolysed with α -chymotrypsin, pepsin, Nagarse, Proctase A or Proctase B. The amino acid sequences of the fragment peptides were determined by subtractive Edman degradation. 2. From the tryptic digest of reduced, *S*-carboxymethylated and trifluoroacetylated erabutoxin b two fragments were isolated. From the amino acid composition of the fragments and from the terminal sequence studies on the reduced and *S*-carboxymethylated erabutoxin b, the sequence of the above seven tryptic fragments was elucidated. 3. The tryptic digestion of reduced and *S*-carboxymethylated erabutoxin a gave fragments, only one of which was different from the corresponding fragment from erabutoxin b. The amino acid sequence analysis of the fragment peptide showed that the only difference between erabutoxins a and b was that the former had asparagine and the latter had histidine at position 26.

Erabutoxins a and b are neurotoxic proteins of a sea-snake (*Laticauda semifasciata*) venom (Tamiya & Arai, 1966; Sato, Yoshida, Abe & Tamiya, 1969). Each of them consists of 62 amino acid residues with arginine at the *N*-terminus. The results of amino acid analysis showed that erabutoxins a and b had the same amino acid composition except that the former had one more aspartic acid residue and one less histidine residue than the latter. The present paper describes the amino acid sequences of erabutoxins a and b.

Similar neurotoxic proteins were isolated from the venoms of other species of sea snakes (laticotoxin a from *Laticauda laticaudata* and *Laticauda colubrina*; Sato *et al.* 1969) and those of cobras. The amino acid sequences of some cobra toxins, namely toxin α of *Naja nigricollis* (Eaker & Porath, 1967), toxin α of *Naja haje haje* (Botes & Strydom, 1969) and cobrotoxin of *Naja naja atra* (Yang, Yang & Huang, 1969) have been elucidated. It is noteworthy that the sea-snake and cobra toxins have a common feature in their structures.

EXPERIMENTAL

Erabutoxins a and b. Erabutoxins a and b were prepared as described by Tamiya & Arai (1966) and desalted by gel filtration (Sato *et al.* 1969).

Proteases. Trypsin (twice crystallized and salt-free), α -chymotrypsin (three-times crystallized), pepsin (twice crystallized) and leucine aminopeptidase were the pro-

ducts of Worthington Biochemical Corp., Freehold, N.J., U.S.A. Nagarse, a crystalline bacterial alkaline protease, was obtained from Nagase Sangyo Ltd., Osaka, Japan. Proctases A and B, acid proteases produced by *Aspergillus niger* var. *macrosporus* (Yamasaki, Shiraki, Horiuchi, Yamada & Goi, 1967), were a gift from Dr Yamasaki. Carboxypeptidase A was given by Dr T. Tobita of Chiba University.

Reagents. Phenylisothiocyanate was synthesized from aniline, carbon disulphide and ammonia (Dains, Brewster & Olander, 1941). Ethyltrifluoroethioacetate was synthesized from trifluoroacetic acid anhydride and ethanethiol (Hauptschein, Stokes & Nodiff, 1952).

Monoiodoacetic acid was recrystallized from chloroform. 2-Mercaptoethanol was redistilled under reduced pressure before use. Pyridine, benzene, diethyl ether and triethylamine were redistilled and other solvents and reagents were used without further purification.

Preparation of RCM-Ea* and RCM-Eb. The reduction and *S*-carboxymethylation of erabutoxins a and b were performed as described by Crestfield, Moore & Stein (1963). The final reaction mixture (6.5 ml) was applied to a Sephadex G-25 (coarse grade) column (2.8 cm $\times$ 65 cm) that had been equilibrated with 10% (v/v) acetic acid and wrapped with aluminium foil. The RCM-Eb, which was eluted with the 10% (v/v) acetic acid at the void volume,

* Abbreviations: Ea and Eb (in names of derivatives), erabutoxins a and b respectively; RCM-, reduced and *S*-carboxymethylated; TFA-, trifluoroacetyl; Cmc (in amino acid sequences), *S*-carboxymethylcysteine; pGlu (in amino acid sequences), pyrrolidonecarboxylic acid (pyroglutamic acid).

was freeze-dried. About 105 mg of RCM-Eb was obtained from 120 mg of erabutoxin b. RCM-Ea was prepared in the same way.

Tryptic digestion and the separation of the resulting peptides. Trypsin was dissolved in 0.05 M-tris chloride buffer, pH 8.2, and treated with diphenylcarbamoyl chloride (Erlanger, Cooper & Cohen, 1966). A portion of the solution containing 1 mg of trypsin was added to an RCM-Eb solution (99 mg of RCM-Eb in 10 ml of 0.1 M-sodium phosphate buffer, pH 7.8). After 7 h at room temperature the mixture was adjusted to pH 2.2 with 1 M-HCl and applied to a Dowex 50 column. Ninhydrin-positive fractions were freeze-dried.

Preparation and tryptic digestion of TFA-RCM-Eb. The trifluoroacetylation was carried out as described by Goldberger & Anfinsen (1962). The resulting TFA-RCM-Eb was precipitated from the reaction mixture by acidification to pH 3.5 with acetic acid, collected by centrifugation, washed with ethanol (five 10 ml portions) and dried *in vacuo*. About 26 mg of the TFA-RCM-Eb was obtained from 35 mg of erabutoxin b.

The TFA-RCM-Eb (26 mg) was dissolved in water (3 ml) and the pH of the solution adjusted to 7.8 with 0.01 M-KOH. The solution was added with the above diphenylcarbamoyl chloride-treated trypsin solution containing 0.3 mg of trypsin. After 4 h at room temperature the solution was freeze-dried and the residue treated with 1 M-piperidine solution (2 ml) to remove the trifluoroacetyl groups. The freeze-dried residue of the mixture was dissolved in 0.1 M-acetic acid (2 ml) and applied to a Sephadex G-25 column (2.8 cm $\times$ 35 cm) equilibrated with 0.1 M-acetic acid. The column was eluted with the same acetic acid solution at a flow rate of 40 ml/h. The first effluent absorbing at 230 nm was collected and freeze-dried. The peptides in the dried residue were further purified by paper electrophoresis at pH 3.7 and 6.5.

Paper electrophoresis and paper chromatography. The paper electrophoresis was carried out on no. 50 filter paper of Toyo Roshi Co., Tokyo, Japan, with pyridine-acetic acid-water (1:10:289, by vol., for pH 3.7, or 25:1:225, by vol., for pH 6.5) or with 0.1 M-triethylamine-acetate buffer, pH 9.0 (0.1 M-triethylamine solution adjusted to pH 9.0 with acetic acid). A voltage gradient of 50 V/cm was applied for a suitable period. The paper chromatography was run with butan-1-ol-acetic acid-water (4:1:2, by vol.) at room temperature for 24 h by the ascending method.

The paper was dried in the air and the parts carrying peptides were cut out by the aid of guiding strips, which were sprayed with ninhydrin or other reagents. The peptides were eluted from the paper with 0.2 M-acetic acid or with 0.1 M-ammonia and freeze-dried.

Further enzymic digestion of tryptic peptides. Some of the tryptic peptides from RCM-Eb or RCM-Ea were further digested to smaller fragments with other proteases. The digestion with Nagarse or α -chymotrypsin was performed in 0.1 M-triethylamine-bicarbonate buffer, pH 8.2, at room temperature for 16 h. The pepsin and Proctase (A or B) digestions were made in 0.01 M-HCl and in 1.0 M-formic acid respectively at 37°C for 16 h. The enzyme/substrate ratio was 1:20–30 (w/w).

Amino acid sequence analysis. (1) Dinitrophenylation. About 0.1–0.5 μ mol of protein or peptide was dinitrophenylated as described by Fraenkel-Conrat, Harris & Levy

(1955b) and hydrolysed with 6 M-HCl (0.5 ml) in an evacuated sealed glass tube at 105°C for 16 h. DNP-amino acids in vacuum-dried hydrolysate were identified by t.l.c. on silica gel (Wako gel B-5; Wako Pure Chemicals Co. Ltd., Tokyo, Japan). The solvents used were: I, chloroform-2-methylbutan-2-ol-acetic acid (70:30:3, by vol.); II, benzene-pyridine-acetic acid (40:10:1, by vol.). For quantitative analysis DNP-amino acids were separated by paper chromatography with 2-methylbutan-2-ol saturated with 0.1 M-phosphate buffer, pH 6.0, as solvent or by paper electrophoresis in 0.1 M-ammonia (for DNP-arginine and ϵ -DNP-lysine). The DNP-amino acids were eluted from the paper with 4% (w/v) NaHCO₃ and determined by spectrophotometry at 340 nm.

(2) Edman degradation. The amino acid sequence of each peptide was determined by subtractive Edman degradation (Konigsberg, 1967) on 0.02–0.2 μ mol samples. A portion of the remaining peptide was subjected to amino acid analysis after the acid hydrolysis (see below) and the rest to another cycle of Edman digestion.

(3) Carboxypeptidase A or leucine aminopeptidase digestion. The digestion was carried out in 0.05 M-tris chloride buffer, pH 7.5, at 37°C for 16 h with 0.01–0.05 μ mol of peptide as the substrate. The enzymes were pretreated with di-isopropyl phosphorofluoridate (Hill & Smith, 1957; Fraenkel-Conrat, Harris & Levy, 1955a) and used at an enzyme/substrate ratio of 1:20–50 (w/w). The digests were directly subjected to amino acid analysis. In some cases the amino acids released were identified by the DNP method.

(4) Tritium labelling of C-terminal amino acid. The C-terminal amino acids of erabutoxins a and b were determined by the tritiation method of Matsuo, Fujimoto & Tatsuno (1966). Erabutoxin a (2.6 mg) was dissolved in tritiated water (0.1 ml; 75 mCi) and kept at room temperature for 4 h. The mixture was dried *in vacuo* and, after the addition of water (0.5 ml), dried again. The final step was repeated again to remove tritiated water. The residue was hydrolysed with 6 M-HCl (0.5 ml) in an evacuated sealed glass tube at 105°C for 16 h. The hydrolysate was dried *in vacuo* and subjected to paper chromatography with as solvent butan-1-ol-acetic acid-water (4:1:1, by vol.) or phenol-water (5:1, v/v). The dried paper strip was cut into pieces (5 mm wide) and each piece counted for radioactivity, to detect the C-terminal amino acid, in a low-background gas-flow counter (Aloka LBC-20; Nihon Musen Co., Tokyo, Japan). The C-terminal amino acid of erabutoxin b was detected in the same way.

Amino acid analysis. The peptides (0.01–0.1 μ mol) were hydrolysed with 6 M-HCl (0.5 ml) at 105°C for 20–24 h in evacuated sealed glass tubes. The hydrolysates were dried *in vacuo* and analysed with automatic amino acid analysers (Mitamura type II from Mitamura Riken Co., Tokyo, Japan; Hitachi KLA-2 from Hitachi Ltd., Tokyo, Japan; JLC-5AH from Japan Electron Optics Ltd., Tokyo, Japan). The enzymic digests were dried *in vacuo* and analysed in the same way. Asparagine and glutamine appeared superposing with serine and threonine respectively.

Preparation of 'fingerprint maps' of tryptic digests. The tryptic digestion mixture of RCM-Eb (40 μ l) described above or that of RCM-Ea was applied on a sheet (40 cm $\times$ 40 cm) of no. 50 filter paper (Toyo Roshi Co.) and subjected to paper electrophoresis in 0.1 M-pyridine-acetate

buffer, pH 4.8, at 40V/cm for 90min followed by paper chromatography at right-angles with butan-1-ol-acetic acid-water (4:1:2, by vol.) for 28h by the ascending method. Peptides were located on the paper by spraying with 1% (w/v) ninhydrin in butan-1-ol. Tryptophan, tyrosine or arginine in the peptides was detected with Ehrlich reagent (Smith, 1953), nitrosonaphthol reagent (Acher & Crocker, 1952) or Sakaguchi reagent (Sakaguchi, 1950).

RESULTS

Terminal residues

Arginine was detected as the *N*-terminal amino acid by direct dinitrophenylation of both erabutoxin a and erabutoxin b (Tamiya & Arai, 1966). The recovery of the DNP-arginine from RCM-Eb was 48% of the theoretical value. The second amino acid from the *N*-terminus of erabutoxin b was determined to be isoleucine (or leucine) by Edman degradation and subsequent dinitrophenylation.

Asparagine (or aspartic acid; Holcomb, James & Ward, 1968) was detected as the *C*-terminal amino acid of both erabutoxin a and erabutoxin b by the tritiation method. On carboxypeptidase A digestion (enzyme/substrate ratio 1:50, w/w) 0.6mol of asparagine was released/mol of erabutoxin b. Thus the terminal residues of erabutoxin b are Arg-Ile(or Leu)...Asn.

Separation of tryptic peptides of RCM-Eb

The elution pattern of the tryptic digests of RCM-Eb from a Dowex 50 column is shown in Fig. 1. The peptides from peaks T_1 , T_3 and T_5 were pure on paper chromatography and paper electro-

phoresis. The peptides from peaks T_2 and T_4 were separated into peptides T_{2a} and T_{2b} and peptides T_{4a} and T_{4b} respectively by paper electrophoresis at pH 6.5. Peptide T_{4a} was ninhydrin-negative and Sakaguchi-positive. The amino acid compositions of the tryptic peptides are given in Table 1. The amino acid composition of erabutoxin b can be explained by these peptides.

Amino acid sequences of tryptic peptides of RCM-Eb

Peptide T_1 (*Leu-Ser-Cmc-Cmc-Glu-Ser-Glu-Val-Cmc-Asn-Asn*). This is the *C*-terminal peptide since it contains neither a lysyl nor an arginyl residue. DNP-leucine was detected on dinitrophenylation. Carboxypeptidase A digestion yielded asparagine (1.41 mol), *S*-carboxymethylcysteine (0.30 mol) and valine (0.10 mol)/mol of peptide T_1 . The *C*-terminal asparagine agrees with that of whole erabutoxin b or RCM-Eb. The partial structure of peptide T_1 is therefore Leu(Ser₂,Cmc₂,Glu₂,Val)Cmc-Asn-Asn.

Four peptides, T_1 -P₁, T_1 -P₂, T_1 -P₃ and T_1 -P₄, were isolated from the peptic digests of peptide T_1 by paper electrophoreses at pH 3.7 and 6.5.

Peptide T_1 -P₁ (*Leu-Ser-Cmc*). This is the *N*-terminal peptide of peptide T_1 because it contains the only and *N*-terminal leucine residue of that peptide. Amino acid analysis showed that it was (Leu,Ser,Cmc). Two steps of subtractive Edman degradation showed that the *N*-terminal sequence was Leu-Ser-.

Peptide T_1 -P₂ (*Cmc-Glu-Ser-Glu*). Amino acid analysis showed that the peptide was (Cmc,Ser,Glx,-Glx). Three steps of subtractive Edman degradation showed that the *N*-terminal structure was

Fig. 1. Elution pattern of tryptic peptides of RCM-Eb from a Dowex 50 column. About 11 ml of tryptic digest (adjusted to pH 2.2) of RCM-Eb (99 mg) was applied to a Dowex 50 (X2; 200–400 mesh) column (0.9 cm $\times$ 30 cm) that was maintained at 37°C with a constant-temperature jacket and had been equilibrated with 0.2M-pyridine-acetate buffer, pH 3.3, and eluted with the same buffer. At the point indicated by the arrow, the elution was continued with a linear gradient to 1.0M-pyridine-acetate buffer, pH 5.3 with a mixing chamber of 600 ml at the beginning. Fractions (2 ml) were collected at a flow rate of 20 ml/h and a 0.05 ml sample of each was checked for ninhydrin reaction after alkaline hydrolysis (Yemm & Cocking, 1955). $\circ$, E_{540} ; ----, pH.

Table 1. *Amino acid composition of tryptic peptides of RCM-Eb*

Amino acids present to an extent less than 0.13 mol/mol were ignored.

Peptide	T ₁	T _{2a}	T _{2b}	T ₃	T _{4a}	T _{4b} †	T ₅	Total	Erabutoxin b‡
Cmc	2.30 (3)	1.60 (2)		1.40 (2)			0.77 (1)	8	8
Asp	1.70 (2)				0.96 (1)		1.00 (1)*	4	4
Thr		0.84 (1)		1.00 (1)		0.75 (1)	1.85 (2)	5	5
Ser	1.80 (2)			2.70 (3)	0.95 (1)		1.95 (2)	8	8
Glu	1.70 (2)			1.00 (1)*	1.00 (1)*	1.00 (1)*	2.99 (3)	8	8
Pro		1.80 (2)		1.26 (1)			1.35 (1)	4	4
Gly		2.85 (3)		1.22 (1)		0.89 (1)		5	5
Ala								0	0
Val	0.78 (1)	0.95 (1)						2	2
Met								0	0
Ile		1.00 (1)*				1.81 (2)	0.86 (1)	4	4
Leu	1.00 (1)*							1	1
Tyr				0.72 (1)				1	1
Phe					1.03 (1)		0.96 (1)	2	2
Lys		2.20 (2)		1.06 (1)			0.92 (1)	4	4
His				1.15 (1)			0.92 (1)	2	2
Arg			1 (1)*		0.91 (1)	1.12 (1)		3	3
Trp					1 (1)§			1	1
Total	11	12	1	12	6	6	14	62	62
Yield (% of theoretical)	85	55	65	50	48	80	80		

* The number of these amino acids were assumed to be 1.00 as the standard.

† The acid hydrolysis was carried out at 110°C.

‡ Taken from Sato *et al.* (1969).§ Determined by the *p*-dimethylaminobenzaldehyde method (Spies & Chambers, 1948).

Cmc-Glx-Ser-. Leucine aminopeptidase and carboxypeptidase A digestion yielded *S*-carboxymethylcysteine, serine and glutamic acid but no glutamine.

Peptide T₁-P₃ (Val-Cmc-Asn-Asn). The amino acid analysis of the peptide showed that it was (Cmc,Asx,Asx,Val). This is the *C*-terminal peptide of peptide T₁ and consequently the *C*-terminal peptide of the erabutoxin b molecule, because it contains two asparagine residues at the terminus. DNP-valine was detected from the acid hydrolysate of the dinitrophenylated peptide. Leucine aminopeptidase digestion of the peptide produced valine, *S*-carboxymethylcysteine and asparagine in the molar proportions 1.0:0.9:0.8. The *N*-terminal sequence is therefore Val-Cmc-.

Peptide T₁-P₄ (Leu-Ser-Cmc-Cmc-Glu-Ser-Glu). The amino acid composition of the peptide showed that it consisted of peptides T₁-P₁ and T₁-P₂. On leucine aminopeptidase digestion the peptide liberated leucine, serine, *S*-carboxymethylcysteine and glutamic acid in the molar proportions 1.0:1.3:1.7:1.4.

Peptide T_{2a} (Gly-Cmc-Gly-Cmc-Pro-Thr-Val-Lys-Pro-Gly-Ile-Lys). Amino acid analysis of the peptide showed that it contained two lysyl residues; one of the two lysyl bonds resisted the trypsin

treatment. Glycine was found to be the *N*-terminal amino acid by dinitrophenylation. Application of three steps of subtractive Edman degradation gave the *N*-terminal structure Gly-Cmc-Gly-.

Hydrolysis of peptide T_{2a} with Nagarse yielded four peptides, T_{2a}-N₁, T_{2a}-N₂, T_{2a}-N₃ and T_{2a}-N₄, which were separated from each other by paper electrophoresis at pH 6.5. The amino acid composition of peptide T_{2a} can be explained as the sum of these four peptides.

Peptide T_{2a}-N₁ (Gly-Cmc). The peptide contained *S*-carboxymethylcysteine and glycine. Dinitrophenylation of the peptide yielded only DNP-glycine.

Peptide T_{2a}-N₂ (Gly-Cmc-Pro-Thr). Amino acid analysis of the peptide showed that it was (Cmc,Thr,-Pro,Gly). Three steps of subtractive Edman degradation on the peptide gave the *N*-terminal structure Gly-Cmc-Pro-. The glycine at the *N*-terminus, which was detected also by dinitrophenylation, explains the third amino acid of peptide T_{2a}. The peptide sequence T_{2a}-N₁-T_{2a}-N₂, can be ascribed to *N*-terminal part of peptide T_{2a}.

Peptide T_{2a}-N₃ (Val-Lys-Pro-Gly). The peptide consisted of equal molar proportions of these four amino acids. DNP-valine was detected on dinitrophenylation. Three steps of subtractive Edman

degradation showed that the *N*-terminal structure was Val-Lys-Pro-. The lysyl-prolyl bond resisted the trypsin treatment.

Peptide T_{2a} - N_4 (Ile-Lys). Amino acid analysis showed that the peptide was (Ile,Lys). DNP-isoleucine only was detected on dinitrophenylation. This peptide represents the *C*-terminal part of peptide T_{2a} , because it has lysine at the end.

Peptide T_{2b} (Arg). Arginine was the only amino acid found in the acid hydrolysate. The electrophoretic mobility of peptide T_{2a} was the same as that of arginine.

Peptide T_3 (Thr-Cmc-Pro-Ser-Gly-Ser-Glu-Ser-Cmc-Tyr-His-Lys). The application of five steps of subtractive Edman degradation on the peptide showed that the *N*-terminal structure was Thr-Cmc-Pro-Ser-Gly-. *N*-Terminal threonine was detected also by the dinitrophenylation method.

Two peptides, T_3 - C_1 and T_3 - C_2 , were separated from the chymotryptic digest of peptide T_3 by paper electrophoresis at pH 6.5. The amino acid compositions of the peptides showed that peptide T_3 was split into two parts.

Peptide T_3 - C_1 (Thr-Cmc-Pro-Ser-Gly-Ser-Glu-Ser-Cmc-Tyr). This peptide is the *N*-terminal part of peptide T_3 , because it contains the only and *N*-terminal threonine of peptide T_3 and no basic amino acid. On carboxypeptidase A treatment the peptide produced tyrosine, *S*-carboxymethyl-cysteine and serine in the molar proportions 1.0:0.39:0.20. When the enzyme treatment was shortened to 1 h, tyrosine only was detected. The partial structure of peptide T_3 - C_1 is, so far, Thr-Cmc-Pro-Ser-Gly(Ser,Glu)Ser-Cmc-Tyr.

A further digestion of peptide T_3 - C_1 with Proctase B produced three peptides, one of which (T_3 - C_1 -Pr) was isolated in pure state by paper electrophoreses at pH 3.7 and 6.5. Amino acid analysis showed that it was (Ser,Ser,Glx,Gly). Subtractive Edman degradation of the peptide gave the *N*-terminal structure Gly-Ser-Glx-. The peptide Gly-Ser-Glx-Ser, containing the only glutamic acid (or glutamine) residue of peptide T_3 - C_1 , explains the middle part of peptide T_3 - C_1 . Peptide T_3 - C_1 -Pr migrated towards the anode (5.2 cm at 50 V/cm for 30 min) on electrophoresis at pH 6.5, showing that the Glx residue was glutamic acid.

Peptide T_3 - C_2 (His-Lys). Amino acid analysis showed that the peptide was (Lys,His). Histidine was detected as the bis-DNP derivative by dinitrophenylation. Having lysine at the end, the peptide explains the *C*-terminal part of peptide T_3 .

Peptide T_{4a} (pGlu-Trp-Ser-Asp-Phe-Arg). The peptide was ninhydrin-negative and Ehrlich- and Sakaguchi-positive. The u.v.-absorption curve of the peptide showed that it contained tryptophan.

On chymotryptic digestion peptide T_{4a} gave two peptides, T_{4a} - C_1 and T_{4a} - C_2 , which were separated

from each other by paper electrophoresis at pH 6.5.

Peptide T_{4a} - C_1 (pGlu-Trp). The peptide was acidic (migrated towards the anode for 8.4 cm at 50 V/cm for 30 min on paper electrophoresis at pH 6.5), ninhydrin-negative and Ehrlich-positive. Glutamic acid was the only amino acid detected by the automatic analyser. The tryptophan content of the peptide, estimated by the *p*-dimethylaminobenzaldehyde method (Spies & Chambers, 1948), was 0.8 mol/mol of glutamic acid. It was thus concluded that peptide T_{4a} - C_1 was pGlu-Trp and that it came from *N*-terminus of peptide T_{4a} .

Peptide T_{4a} - C_2 (Ser-Asp-Phe-Arg). Amino acid analysis and the application of three steps of subtractive Edman degradation of the peptide disclosed the above structure. The *N*-terminal serine was detected also by dinitrophenylation. The peptide was neutral (migrated towards the cathode for 1.2 cm at 50 V/cm for 30 min on paper electrophoresis at pH 6.5) and aspartic acid was found in the leucine aminopeptidase digest of peptide T_{4a} . It was therefore concluded that the Asx residue in peptide T_{4a} - C_1 or in peptide T_{4a} was aspartic acid.

Peptide T_{4b} (Gly-Thr-Ile-Ile-Glu-Arg). The peptide was neutral and appeared yellow on paper after ninhydrin. Four steps of subtractive Edman degradation on the peptide showed that the *N*-terminal structure was Gly-Thr-Ile-Ile-. Glutamic acid was released by leucine aminopeptidase digestion of the peptide.

Peptide T_5 (Ile-Cmc-Phe-Asn-Gln-His-Ser-Ser-Gln-Pro-Gln-Thr-Thr-Lys). Four steps of subtractive Edman degradation of peptide T_5 showed that the *N*-terminal structure was Ile-Cmc-Phe-Asx-. The *N*-terminal isoleucine was detected also by dinitrophenylation.

Digestion of peptide T_5 with Nagarse produced four peptides, T_5 - N_1 , T_5 - N_2 , T_5 - N_3 and T_5 - N_4 . Peptides T_5 - N_1 and T_5 - N_2 were isolated by paper electrophoresis at pH 6.5 and the mixture of peptides T_5 - N_3 and T_5 - N_4 was further separated to its components by paper electrophoresis at pH 9.0. The amino acid compositions of the four peptides explain the total amino acid composition of peptide T_5 .

Peptide T_5 - N_1 (Ile-Cmc-Phe-Asn). This is the *N*-terminal peptide from peptide T_5 , because it contains the only and *N*-terminal isoleucine of peptide T_5 . Hydrolysis of peptide T_5 - N_1 with carboxypeptidase A liberated asparagine and phenylalanine in the molar ratio 1.0:0.9. The above sequence was concluded from the results of subtractive Edman degradation of peptide T_5 described above.

Peptide T_5 - N_2 (Ser-Ser-Gln-Pro-Gln). Serine was detected as the *N*-terminal residue by dinitrophenylation. Four steps of subtractive Edman degradation on the peptide showed that the *N*-

terminal structure was Ser-Ser-Glx-Pro-. The peptide was neutral (migrated towards the cathode for 1.0 cm at 50 V/cm for 30 min on paper electrophoresis at pH 6.5) and no glutamic acid was detected in its leucine aminopeptidase digest, which contained free proline. It was therefore concluded that two Glx residues were glutamine residues.

Peptide T_5-N_3 (pGlu-His). The peptide was ninhydrin-negative, Pauli-positive and consisted of only glutamic acid and histidine. The peptide was ninhydrin-positive and basic on the first paper electrophoresis at pH 6.5 and turned ninhydrin-negative and neutral on the second paper electrophoresis in triethylamine buffer, pH 9.0. It was thus concluded that the peptide was Gln-His and cyclized to pGlu-His at pH 9.0.

Peptide T_5-N_4 (Thr-Thr-Lys). This is the C-terminal peptide of peptide T_5 , because it contains the only lysine residue of peptide T_5 , a tryptic peptide from RCM-Eb. The sequence is clear from the specificity of trypsin.

From the Proctase A digest of peptide T_5 a peptide, T_5 -Pr, containing histidine was isolated by paper electrophoresis at pH 3.7. The amino acid composition of the peptide showed that the peptide was (Asx, Ser, Ser, Glx, His). The composition can be explained assuming the peptide sequence $T_5-N_1-T_5-N_3-T_5-N_2$, which gives the amino acid sequence Asn-Gln-His-Ser-Ser for peptide T_5 -Pr.

Peptide sequence in erabutoxin b

A partial peptide sequence $T_{2b}-T_5-(T_{2a}, T_3, T_{4a}-T_{4b})-T_1$ is concluded from the terminal-structure studies on RCM-Eb, described above.

Two major peptides were purified from the tryptic digest of TFA-RCM-Eb, as described in the Experimental section. The amino acid compositions of the peptides (peptides A and B) are given in Table 2. The amino acids of peptide A can be explained as the sum of those of peptides T_{2a} and T_1 , and those of peptide B as the sum of those of peptides T_5 , T_3 and T_{4a} . The sequence $T_5-T_3-T_{4a}$ in peptide B is clear because arginine must be placed at the C-terminus and peptide T_5 is next to peptide T_{2b} at the N-terminus. Peptide T_{4b} , which is contained neither in peptide A nor in peptide B, must be placed between peptides A and B.

It was thus concluded that RCM-Eb has the peptide sequence $T_{2b}-T_5-T_3-T_{4a}-T_{4b}-T_{2a}-T_1$.

Amino acid sequence of erabutoxin a

Erabutoxin a was reduced, carboxymethylated and digested with trypsin as described in the Experimental section. A comparison of 'fingerprint maps' of tryptic peptides from RCM-Ea and RCM-Eb showed that seven spots out of eight on each 'map' are common to both (Fig. 2). Specific colour

Table 2. Amino acid composition of tryptic peptides of TFA-RCM-Eb

Peptide	A	B
Cmc	4.70 (5)	1.95 (3)†
Asp	2.00 (2)*	2.00 (2)*
Thr	0.96 (1)	2.50 (3)
Ser	1.70 (2)	6.60 (6)
Glu	2.08 (2)	5.00 (5)
Pro	2.12 (2)	2.07 (2)
Gly	3.10 (3)	0.90 (1)
Ala	0	0
Val	1.69 (2)	0
Met	0	0
Ile	1.06 (1)	0.70 (1)
Leu	1.06 (1)	0
Tyr	0	0.63 (1)
Phe	0	1.70 (2)
Lys	2.00 (2)	2.10 (2)
His	0	2.10 (2)
Arg	0	1.40 (1)
Trp	—	+‡
Total	23	32

* The number of aspartic acid was assumed to be 2.00 as the standard.

† S-Carboxymethylcysteine tends to give too small value.

‡ Detected by u.v. absorption.

reactions and amino acid analyses of the spot peptides revealed that the common peptides were T_1 , T_{2a} , T_{2b} , T_{4a} (ninhydrin-negative), T_{4b} , T_5 and T_{4a}' . Peptide T_{4a}' had the same amino acid composition as peptide T_{4a} but was ninhydrin-positive. It was therefore concluded that peptide T_{4a}' was the precursor of peptide T_{4a} with uncyclized glutamine at the N-terminus. In the preparation of peptide T_{4a} , described above, the cyclization must have occurred on Dowex-50 treatment (Blombäck, 1967). The only peptide that was not common, T_{3Ea} , contained tyrosine, as did peptide T_3 from RCM-Eb.

Peptide T_{3Ea} was hydrolysed with α -chymotrypsin. Two peptides, $T_{3Ea}-C_1$ and $T_{3Ea}-C_2$, were isolated from the hydrolysate by paper electrophoresis at pH 6.5. The amino acid composition of peptide T_{3Ea} was explained by these peptides. The amino acid composition of peptide $T_{3Ea}-C_1$ agreed with that of peptide T_3-C_1 described above. Peptide $T_{3Ea}-C_2$, which consisted of equimolar aspartic acid and lysine, corresponds to peptide T_3-C_2 , i.e. His-Lys, of RCM-Eb and is placed at the C-terminal part of the original tryptic peptide, T_{3Ea} . Peptide $T_{3Ea}-C_2$ was basic and concluded to have the structure Asn-Lys. The amino acid sequence of peptide T_{3Ea} is therefore, as follows:

The underlined asparaginyl residue is a histidyl residue in peptide T_3 and this is the only difference between erabutoxins a and b.

DISCUSSION

The whole amino acid sequences of erabutoxins a and b are shown in Fig. 3. Out of seven arginyl and lysyl bonds, six were hydrolysed by trypsin,

whereas the lysyl-prolyl bond at position 47 was not. Diphenylcarbamoyl chloride treatment of trypsin completely abolished the chymotrypsin-like activity of the enzyme. The *N*-terminal arginyl-isoleucyl bond was hydrolysed by trypsin.

The amino acid sequence of erabutoxin a is the same as that of erabutoxin b except that histidine-26 of the latter is replaced by asparagine in the former. The replacement can be explained by a single base replacement in the triplet codes (CAC to AAC or CAU to AAU). It is noteworthy that a single specimen of *Laticauda semifasciata* has both erabutoxins a and b in variable ratio, as tested by disc electrophoresis (Tamiya & Arai, 1966).

Some more peptides, which confirm the above structure, were obtained by chemical and enzymic cleavage of erabutoxin b. A tetrapeptide with an amino acid composition (Arg,Ile,Cmc,Phe) (1-4) was isolated from a hydrolysate with 0.03M-hydrochloric acid (Schultz & Grice, 1962) of RCM-Ea. The same peptide and another tetrapeptide (His,Lys,Glx,Trp) (26-29) were isolated from the chymotryptic hydrolysate of RCM-Eb. Three tetrapeptides and a pentapeptide with amino acid compositions (Lys,Gln,Trp,Ser) (27-30), (Asp,Phe,-Arg,Gly) (31-34), (Thr,Ile,Ile,Glu) (35-38) and (Asn,Gln,His,Ser,Ser) (5-9) were obtained from the Proctase A digest of erabutoxin a (Endo, Sato, Ishii & Tamiya, 1971). These results also confirm the peptide sequences in the toxins.

The primary structures of toxins of three species of cobras have so far been elucidated (Fig. 4). Most of the point mutations that occur in the sea-snake and cobra toxins are observed in other related proteins (Dayhoff, 1969). However, the mutations arginine-leucine (1), threonine-tyrosine (35), isoleucine-arginine (36) and valine-arginine (59) are novel.

The 33 underlined amino acid residues among 62 residues are common to all five snake toxins. It is noteworthy that all eight half-cystine residues are at the same positions. The serine residue at position 19 (or glycine residue at position 20) is missing from *Naja haje* and *Naja nigricollis* toxins.

Fig. 2. 'Fingerprint maps' of tryptic digests of RCM-Ea (a) and RCM-Eb (b). Details are given in the text. T_1 , T_{2a} etc. are the peptide spots detected by ninhydrin, Ehrlich reagent, nitrosonaphthol or Sakaguchi reagent spray.

Fig. 3. Amino acid sequences of erabutoxins a and b.

Erabutoxins are known to inhibit the neuromuscular transmission in a similar manner to curare (Tamiya & Arai, 1966). The common basic amino acid residues, namely lysine-27, arginine-33, arginine-37 and lysine-47, may play important roles in blocking the acetylcholine receptor sites on the postsynaptic membrane. The modification of tryptophan-29 brings about loss of the activity (Seto, Sato & Tamiya, 1970). The residue may be involved in the affinity to the membrane.

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