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The Disulphide Bonds of Erabutoxin a, a Neurotoxic Protein of a Sea-Snake (*Laticauda semifasciata*) Venom

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Erabutoxin a was partially hydrolysed with enzymes and sulphuric acid and the resulting peptides were separated from each other by column chromatography and paper electrophoresis. From the results of amino acid analyses of the sulphur-containing peptides and their oxidized components, all four disulphide bridges in the toxin molecule were located. The disulphide bonds were found between half-cystine residues at positions 3 and 24, 17 and 41, 43 and 54, and 55 and 60 from the *N*-terminus.

Erabutoxin a is a crystalline neurotoxic protein obtained from a sea-snake (*Laticauda semifasciata*) venom. There are some similar neurotoxins, namely erabutoxin b and laticotoxin a of sea snakes (Tamiya & Arai, 1966; Sato, Yoshida, Abe & Tamiya, 1969), cobrotoxin (Yang, 1965) and toxins α (Boquet, Izard, Jouannet & Meaume, 1966; Karlsson, Eaker & Porath, 1966; Botes & Strydom, 1969) of cobras. The amino acid sequences of toxin α of *Naja nigricollis* (Eaker & Porath, 1967), toxin α of *Naja haje haje* (Botes & Strydom, 1969), cobrotoxin of *Naja naja atra* (Yang, Yang & Huang, 1969) and erabutoxins a and b of *Laticauda semifasciata* (Sato & Tamiya, 1971) have been elucidated. All five toxins consist of 61 or 62 amino acid residues with eight half-cystine residues among them. All the half-cystine residues are found at corresponding positions of these toxin molecules.

The present study was undertaken to locate the disulphide linkages in the erabutoxin a molecule.

MATERIALS AND METHODS

Erabutoxin a. Erabutoxin a was isolated by the method of Tamiya & Arai (1966), desalted by gel filtration (Sato *et al.* 1969) and freeze-dried.

Proctase A. An acid protease produced by *Aspergillus niger* var. *macrosporus*, named Proctase A, was a kind gift from Dr Yamasaki (Yamasaki, Shiraki, Horiuchi, Yamada & Goi, 1967).

Paper electrophoresis. Paper electrophoresis was carried out on Toyo no. 50 filter paper (Toyo Scientific Instruments, Tokyo, Japan), cooled in chilled hexane at 0–5°C, with solvent mixtures pyridine–acetic acid–water (1:10:89, by vol., for pH 3.6 and 10:7:233, by vol., for pH 5.0) at 40 V/cm. The paper was dried in the air after the electrophoresis and the peptides were located on the paper, if necessary, by spraying with ninhydrin (0.24%, w/v, in acetone), platinochloric acid (Toennies & Kolb, 1951), α-nitrosonaphthol (Acher & Crocker, 1952), Ehrlich reagent (Smith, 1953) or Sakaguchi reagent (Jepson & Smith, 1953). Successive applications of the above sprays were also used (Smith, 1958).

Amino acid analysis. About 0.1 μmol of a peptide was hydrolysed with 6M-HCl (0.3ml) in an evacuated and sealed glass tube at 110°C for 24h. The hydrolysate was dried in an evacuated desiccator and analysed for the amino acid contents with a JLC 5AH automatic amino acid analyser (Japan Electron Optics Laboratory Co., Tokyo, Japan).

EXPERIMENTAL AND RESULTS

Digestion of erabutoxin a with Proctase A and the separation of resulting peptides. Erabutoxin a (42mg) was dissolved in 4.5ml of 1M-formic acid–1M acetic acid (2:1, v/v) mixture and Proctase A (1.2mg) was added. The mixture was incubated at 37°C for 12h and freeze-dried. The number of amino groups, as measured by trinitrobenzenesulphonic acid method (Satake, Okuyama, Ohashi & Shinoda, 1960) was 2.37 times the original value. About 7 new amino groups appeared/molecule of the toxin.

The freeze-dried material was dissolved in 0.1M-formic acid (2.0ml) and passed with the formic acid through a Sephadex G-25 column (2cm × 270cm) that had been equilibrated with the formic acid. The elution pattern as followed by extinction at 230 and 280nm is shown in Fig. 1.

The u.v.-absorbing fractions were collected and freeze-dried. Each one-hundredth part of the dried material was subjected to paper electrophoresis at pH 5.0 for 100min. The results are shown in Fig. 2. The sulphur-containing (platinochloric acid-positive) peptides (shadowed in Fig. 2) were found in peaks II, III and V of Fig. 1.

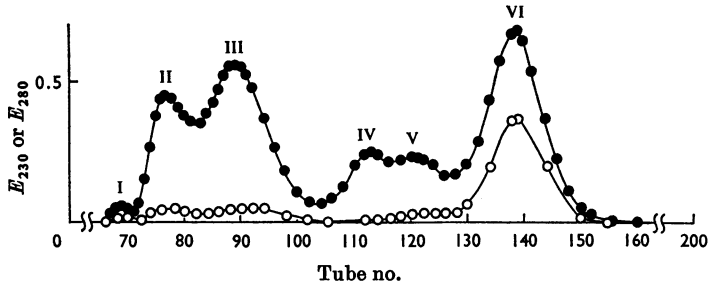

Fig. 1. Sephadex column chromatography of Proctase A digests of erabutoxin a. The Proctase A digests of erabutoxin a were applied on a Sephadex G-25 column (2cm×270cm) and eluted with 0.1M-formic acid. Fractions (each 5ml) were assayed for the extinction at 230nm (●) and 280nm (○) in 1cm cells.

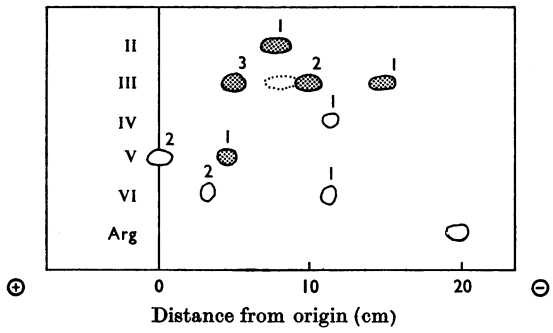

Fig. 2. Paper electrophoresis of Proctase A peptides of erabutoxin a. The Sephadex G-25 fractions of Proctase A digests were subjected to paper electrophoresis at pH5.0 for 100min. The shadowed spots are platinochloric acid-positive.

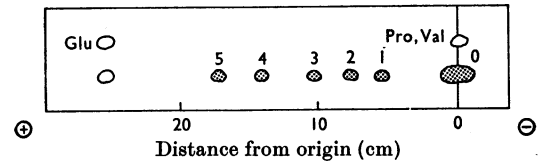

Fig. 3. Paper electrophoresis of sulphuric acid hydrolysate of a Proctase A peptide from erabutoxin a. Peptide III-3, a Proctase A peptide from erabutoxin a, was partially hydrolysed with H_2SO_4 and the hydrolysate subjected to paper electrophoresis at pH5.0 for 4h. The shadowed spots are platinochloric acid-positive.

The remaining main parts of the dried material were subjected to paper electrophoresis under the same conditions, and peptides were oxidized with performic acid vapour on the paper (Brown & Hartley, 1966), extracted with 2.5% (v/v) acetic acid (each 3ml) from the spots and freeze-dried. A part of the sulphur-containing and oxidized peptides were again subjected to paper electrophoresis at pH3.6 and separated into their components. The results of amino acid analyses of the oxidized peptides and their components are shown in Table 1. The results of amino acid analyses of sulphur-free peptides are also shown. Peptide III-2 was separated into three components on the second electrophoresis after the oxidation, whereas peptides III-1 and V-1 were each separated into two components. Peptide III-3 again gave a single component after the oxidation. The amino acids of peptide III-2 can be explained as the sum of those of peptides III-1 and V-1.

Sulphuric acid hydrolysis of a larger fragment. Peptide III-3 (0.8μmol), which contained two disulphide bonds, was partially hydrolysed with 10M-sulphuric acid–acetic acid–water–thioglycollic acid mixture (15:9:6:0.01, by vol.) (0.5ml) in an evacuated and sealed glass tube, for 45min at 100°C (Ryle, Sanger, Smith & Kitai, 1955) and the hydrolysate was applied to a column (1.4cm×5cm) of weakly basic resin (Amberlite CG-4B, OH^- form) to remove the sulphuric acid. The peptides were eluted from the column with 5% (v/v) acetic acid (25ml). The residue obtained by evaporating the eluate at below 32°C in a flash evaporator was dissolved in 2.5% (v/v) acetic acid (0.4ml) and separated into its components by paper electrophoresis at pH5.0 for 4h (Fig. 3). Six spots, out of seven detected by ninhydrin spray, were chloroplatinic acid-positive. Spots 1–5 of the main run of the electrophoresis were oxidized on the paper with performic acid vapour and extracted with 2.5% (v/v) acetic acid (each 3ml). The acetic acid extracts were evaporated to dryness under reduced pressure at 32°C, dissolved in 2.5% (v/v) acetic acid (each 0.2ml) and subjected again to paper electrophoresis at pH3.6. Free cysteic acid was detected as one of the oxidation products from the peptides of

Table 1. *Amino acid compositions of Proctase A peptides and their oxidized components from erabutoxin a*

Abbreviation: Cya, cysteic acid.

	Amino acid composition (mol/mol of peptide)														
	Peptide III-1			Peptide III-2					Peptide V-1						
	Total	Component 1	Component 2	Total	Component 1	Component 2	Component 3	Peptide III-3	Total	Component 1	Component 2	Peptide IV-1	Peptide V-2	Peptide VI-1	Peptide VI-2
Cya	1.82 (2)*	1.27 (1)*	1.34 (1)*	4.15 (4)*	1.04 (1)*	1.16 (1)*	2.02 (2)*	3.94 (4)*	1.82 (2)*	1.01 (1)*	1.21 (1)*				
Asp				0.96 (1)*			0.87 (1)*	2.07 (2)*	0.90 (1)*		0.71 (1)*	1.06 (1)*			1.00 (1)*
Thr	0.86 (1)*		1.06 (1)*	1.23 (1)			1.23 (1)	0.95 (1)					0.94 (1)		
Ser	0.94 (1)*		0.89 (1)*	2.81 (3)			3.13 (3)	1.75 (2)	1.90 (2)		1.54 (2)	1.62 (2)		0.91 (1)	
Glu				1.44 (1)*			1.12 (1)*	2.10 (2)*	1.25 (1)*		0.96 (1)*	1.03 (1)*	1.00 (1)*	1.09 (1)*	
Pro	0.52 (1)		0.96 (1)*	1.17 (1)			1.12 (1)	2.06 (2)							
Gly	2.37 (2)*	1.91 (2)*		3.92 (3)	2.22 (2)*		2.12 (1)	1.14 (1)*	1.37 (1)		1.11 (1)*				0.91(1)*
Val								1.83 (2)*					1.53 (2)		
Ile				0.93 (1)*		1.16 (1)*		0.96 (1)*	1.04 (1)*	1.01 (1)*					
Leu								1.04 (1)*							
Tyr				1 (1)			1 (1)		1 (1)		1 (1)				1.05 (1)*
Phe				0.97 (1)*		0.76 (1)*			0.91 (1)*	0.89 (1)*					
Lys	0.92 (1)		0.74 (1)*	1.02 (1)*			0.98 (1)*	1.89 (2)*						0.90 (1)*	
His												0.91 (1)*			
Arg	1.08 (1)	0.82 (1)*		1.56 (2)*	0.74 (1)*	0.91 (1)*			1.08 (1)*	1.09 (1)*					1.03 (1)*
Trp														1 (1)	
Total	9	4	5	20	4	4	12	20	11	4	7	5	4	4	4

* The total numbers of these amino acids were taken as standard round numbers.

Table 2. *Amino acid compositions of oxidation products of a Proctase A–sulphuric acid peptide from erabutoxin a*

Abbreviation: Cya, cysteic acid.

	Amino acid composition (mol/mol of peptide)	
	Peptide 5a	Peptide 5b
Cya	1.20 (1)*	0.82 (1)*
Asp	0.80 (1)*	
Glu	1.02 (1)*	1.18 (1)*
Val	1.00 (1)*	

* The total numbers of these amino acids were taken as standard round numbers.

spots 1–4. The spot 5 peptide was separated into two components, *5a* and *5b*, after the oxidation. The results of amino acid analysis of the components are shown in Table 2.

DISCUSSION

The amino acid compositions of the peptides presented in Tables 1 and 2 can be explained on the basis of the amino acid sequence of erabutoxin a (Sato & Tamiya, 1971), assuming disulphide linkages between half-cystine residues at positions 3 and 24, 17 and 41, 43 and 54 and 55 and 60 from the *N*-terminus. The structures of the peptides are shown in Fig. 4.

The whole structure of erabutoxin a is shown in Fig. 5. The only difference in the amino acid sequences of erabutoxins a and b is that the latter has histidine at position 26 instead of asparagine in the former. It is therefore reasonable to conclude that erabutoxin b has the same arrangement of disulphide bridges as erabutoxin a.

In addition to the above peptides, the following two peptides were isolated from the Proctases A and B digests of the 'tryptic core' of erabutoxin a:

```
 16  17  18  19
Thr-Cys-Pro-Ser
 40  |   42
Gly-Cys-Gly
     2   3   4
   Ile-Cys-Phe
    23 |   25
   Ser-Cys-Tyr
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The results confirm the above conclusion.

Essentially the same overall arrangement of disulphide bridges was presented at the Second International Symposium on Animal and Plant Toxins held in Tel-Aviv, Israel in February 1970, for cobrotoxin by Dr C. C. Yang and for toxin α by

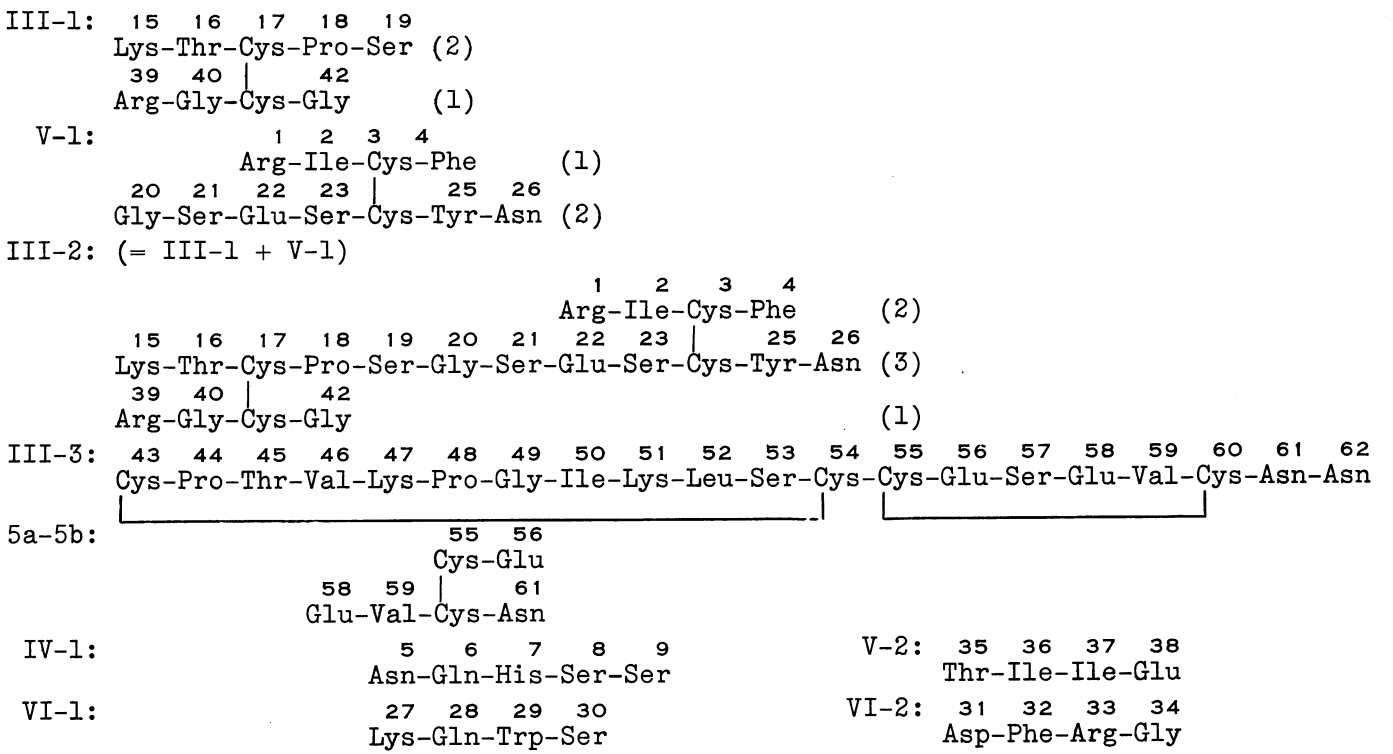

Fig. 4. Structures of the peptides obtained from erabutoxin a.

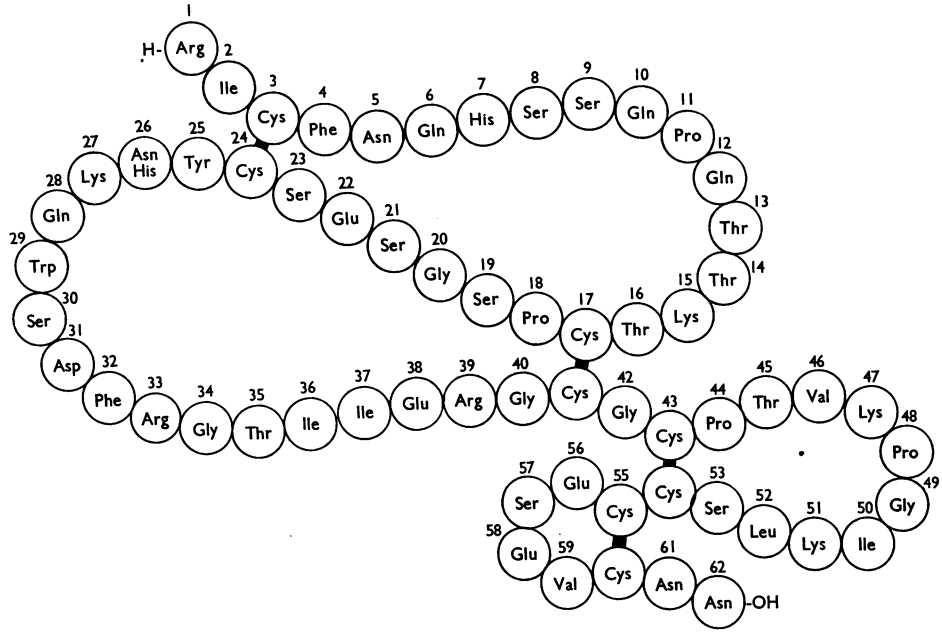

Fig. 5. Structure of erabutoxins a and b. The amino acid at position 26 is asparagine for erabutoxin a and histidine for erabutoxin b.

Dr D. P. Botes. This overall structure therefore seems to be common to neurotoxins of cobras and sea snakes.

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