LOCALIZATION OF THE FOUR DISULFIDE BRIDGES IN CYTOTOXIN II FROM THE VENOM OF THE INDIAN COBRA (Naja naja)*

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SUMMARY

The four disulfide bridges in cytotoxin II from the venom of the Indian cobra have been localized by degradation with thermolysin and by correlation of the disulfide bonds of the cystine peptides so obtained with the corresponding cysteic acid peptides. Cytotoxin II consists of 60 amino acid residues with four intramolecular disulfide bonds, linking half-cystine residues 3 and 21, 14 and 38, 42 and 53, and 54 and 59.

In our studies on the biologically active components of snake venoms, two basic proteins, designated as cytotoxins I and II, were isolated from the venom of the Indian cobra (naja naja) by fractionation on carboxymethyl(CM)-cellulose; recently we determined their amino acid sequences (1, 2, 3). Since the four disulfide bridges determine the secondary structure of the peptide chain and since their integrity is a requirement for toxicity, we have now completed our study on their localization. The four disulfide bonds in cytotoxin II have been established by determining the amino acid composition and sequence of the corresponding cysteic acid peptides from the thermolysin digest and oxidation of cytotoxin II.

MATERIALS AND METHODS

Cytotoxin II was prepared from the venom of the Indian cobra by chromatography on a CM-cellulose column (4). Homogeneity was ascertained by disc gel electrophoresis. Amino acid analyses of the peptides

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obtained by digestion of cytotoxin II with thermolysin and trypsin were carried out as described in previous publications. Digestion with thermolysin (5) was carried out at 37°C in 2% NH_4HCO_3 solution at pH 7.0 for 48 hours in the presence of N-ethylmaleimide at a concentration of 10^{-3}M (6,7). The digest was fractionated on a Sephadex G-25 column (2.2 x 240 cm) in 1% acetic acid. The peptides obtained were further purified by paper chromatography in a solvent system of n-butanol-acetic acid-water, 3:1:1, v/v, and by high voltage paper electrophoresis in a solvent system of pyridine-acetic acid-water, 1:10:289, pH 3.6, v/v. Cystine peptides were developed with cyanide-nitroprusside reagent (8). All cystine peptide bands were cut out from the paper, eluted with 10% acetic acid, and the residues, after evaporation, oxidized with performic acid by adding a few drops of a solution of freshly prepared performic acid by mixing 1 volume of 30% H_2O_2 with 9 volumes of formic acid. Oxidation was allowed to proceed for 30 min, a few drops of water were than added and the solutions were taken to dryness. The residues were subjected to paper electrophoresis at pH 3.6 in order to separate the pure peptides.

RESULTS AND DISCUSSION

The amino acid sequences of neurotoxins isolated from various cobras have been elucidated (5, 9-16). The disulfide bridges in some of these neurotoxins are located at corresponding positions (5, 17, 18). Despite the dissimilarity in their amino acid compositions, the amino acid sequences, the location of cysteine residues and the length of the peptide chains (61 or 62 amino acids) cytotoxins and neurotoxins bear a distinct resemblance to each other. However, cytotoxins show low toxicity when injected intraperitoneally into mice (3).

We considered the possibility that the low toxicity might reflect a difference in the location of the disulfide linkages and therefore analyzed the structures of the cystine containing-peptides obtained by thermolysin digestion. Cytotoxin II was digested with thermolysin

and the digest, dissolved in 1% acetic acid, passed through a Sephadex G-25 column that had been equilibrated with the same solvent. The elution pattern was followed by monitoring extinctions at 230 and 280 nm and is shown in Fig. 1. The u.v. absorbing fractions were collected and freeze-

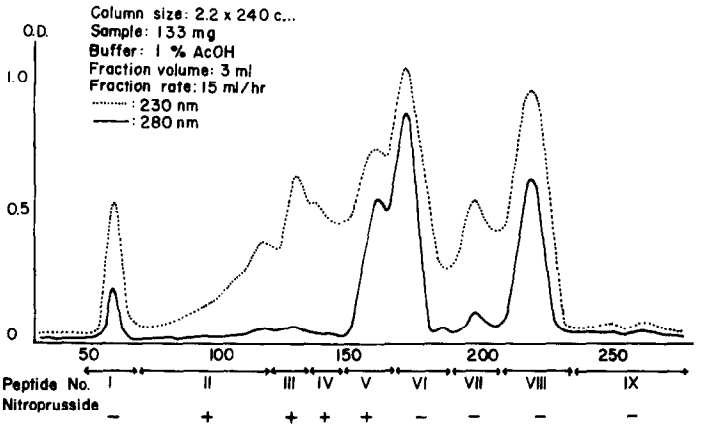

Fig. 1. Gel Filtration of thermolysin digest of cytotoxin II on a column of Sephadex G:25.

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dried. Each fraction was subjected to paper electrophoresis at pH 3.6. The cystine containing-peptides were made visible by cyanide-nitroprusside spray. The areas occupied by cystine peptides were cut out and eluted with 10% acetic acid. The peptides obtained were further purified by descending paper chromatography in a solvent system of n-butanol-acetic acid-water, 3:1:1, v/v. After paper chromatography and paper electrophoresis the peptides with ninhydrin gave single and discrete spots. Each purified peptide was oxidized with performic acid and the reaction mixture subjected to paper electrophoresis at pH 3.6 and separated into individual components. The results of the amino acid analyses of the oxidized peptides and their components are presented in Table I. After performic acid oxidation, Peptide II'' consisted of only one

Table I

Amino Acid Compositions of Disulfide Peptides and Their Oxidized or Tryptic Components (C) from Cytotoxin

	Peptide II'	Peptide II''			Peptide III			Peptide IV			Peptide V		
	Total	Total	C-1	C-2	Total	C-1	C-2	Total	C-1	C-2	Total	C-1	C-2
Lysine	1.0				1.0	1.0		1.0		1.0			
Histidine													
Arginine	1.4	1.0	1.0										
Aspartic Acid	2.6	3.3	2.5	1.0									
Threonine	1.0	1.2	1.0								1.0	0.9	
Serine													
Glutamic Acid													
Proline	1.0				1.0	1.0					1.0	0.9	
Glycine											1.2		1.1
Alanine													
Half-cystine	3.3	1.9			1.4			2.0			1.8		
Valine	2.0												
Methionine													
Isoleucine													
Leucine								2.1	0.9	0.8			
Phenylalanine													
Tyrosine													
Cysteic Acid			1.0	0.9		0.6	1.0		1.0	1.0		1.0	1.0

Numbers represent the nearest integers. The value of the amino acid underlined was taken as 1.0.

component. On the basis of the amino acid composition the peptide represented residues 54 to 59. After cleavage of cytotoxin II with trypsin the peptide digest was separated into two components by paper electrophoresis. The amino acid composition of the peptides presented in Table I can be explained on the basis of the amino acid sequence of cytotoxin II, assuming disulfide bonds between half-cystine residues at positions 3 and 21, 14 and 38, 42 and 53, and 54 and 59. The structures of the peptides are shown in Fig. 2 and the complete sequence of cytotoxin II in Fig. 3.

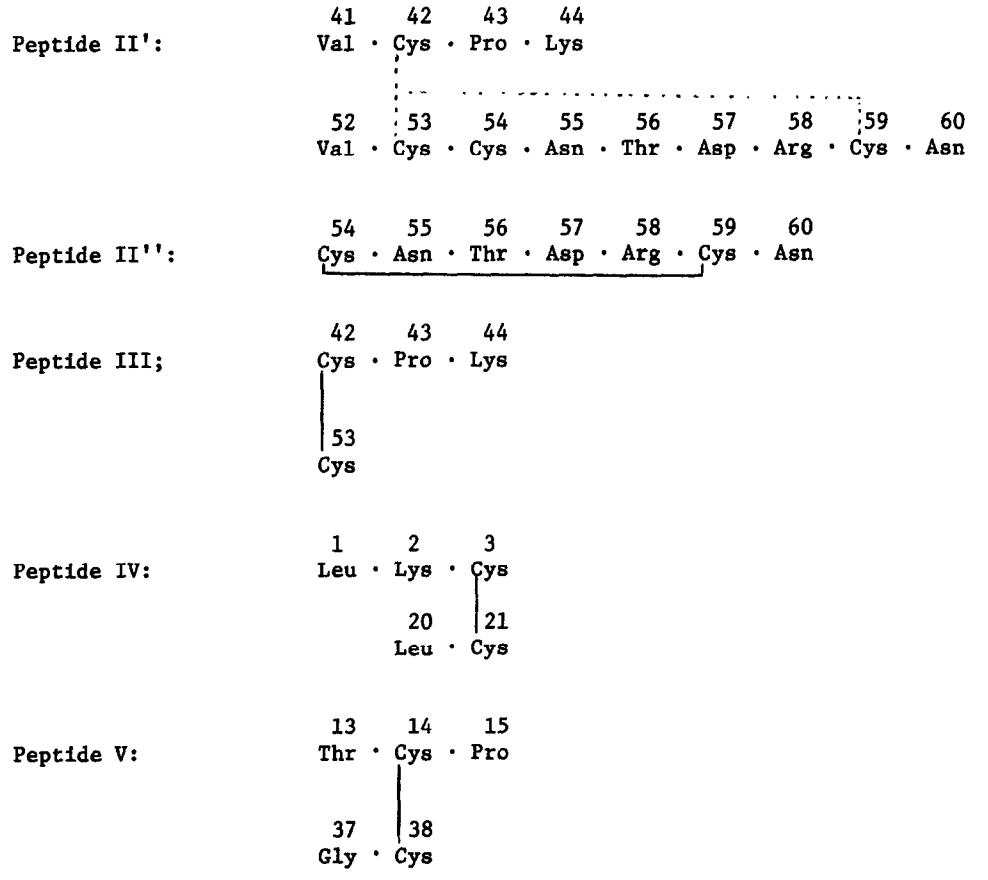

Fig. 2. Proposed sequence of cystine peptides from the thermolysin digest of cytotoxin II.

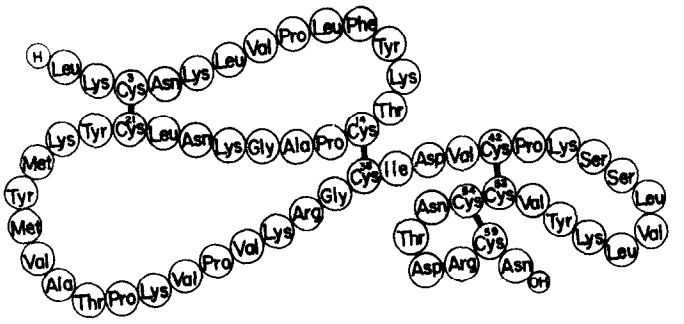

Fig. 3. The amino acid sequence of cytotoxin II showing the positions of the disulfide bridges.

Cytotoxins I and II differ in that the latter has valine, phenylalanine, alanine, threonine, proline, lysine and valine at positions 7, 10, 28, 29, 30, 31 and 52 instead of isoleucine, alanine, serine, aspargine, lysine, threonine and glutamic acid in the former. It is therefore reasonable to conclude that cytotoxin I has the same arrangement of disulfide bridges as cytotoxin II.

There is a remarkable similarity between the positions of the disulfide bridges of cytotoxin II and those of neurotoxins which were recently elucidated. The homologous half-cystine residues are in fact identically cross-linked in neurotoxins consiting of 61 or 62 amino acid residues. Thus, it seems that not so much the position of disulfide bridges determines the degree of toxicity but the amino acid sequence of these venoms.

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