LOCALIZATION OF THE FIVE DISULFIDE BRIDGES IN TOXIN B FROM THE VENOM OF THE INDIAN COBRA (NAJA NAJA)*

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SUMMARY

By degradation with acid protease and thermolysin the five disulfide bridges in toxin B from the venom of the Indian cobra have been localized. Toxin B consists of 71 amino acid residues and the five intramolecular disulfide bridges link half cystine residues 3 and 20, 14 and 41, 26 and 30, 45 and 56, and 57 and 62.

The amino acid sequences of toxins from a variety of species representing the genera Naja (1-8), Haemacutus (9), Laticauda (10, 11) and Dendroaspis (12) have recently been elucidated. In our studies on the biologically active components of snake venom, several toxic proteins, such as toxins A and B, were isolated from the venom of the Indian cobra (Naja naja) by fractionation on carboxymethyl (CM)-cellulose. Recently we established the amino acid sequences of toxins A (7) and B (13). Since the five disulfide bridges determine the tertiary structure of the peptide chain and since their integrity is essential for toxicity, we have now localized the 5 disulfide bridges by determining the amino acid composition and sequence of the corresponding cystic acid peptides obtained from the acid protease and thermolysin digests of toxin B after oxidation with performic acid.

MATERIALS AND METHODS

Toxin B was prepared from the venom of the Indian cobra by chromatography on a CM-cellulose column (14). Homogeneity was ascertained

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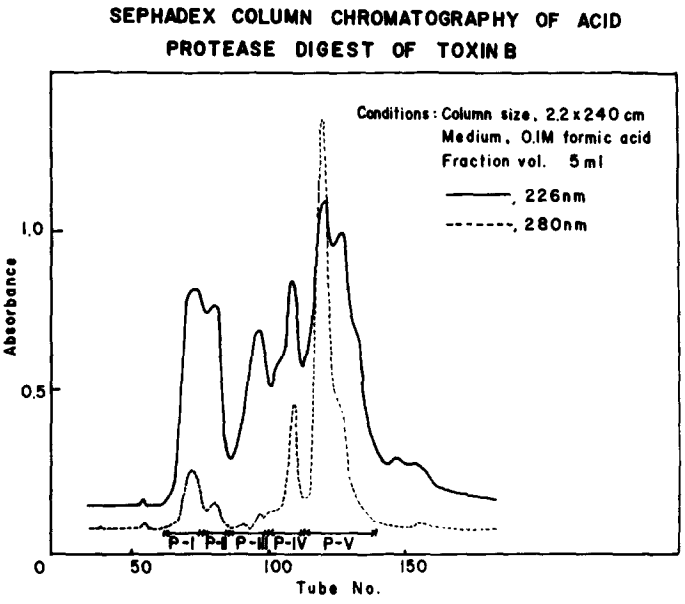

Fig. 1.

by disc gel electrophoresis. Toxin B was digested with acid protease (18) at pH 2.0 in a mixture of 1.0 M formic and 1.0 M acetic acid (2:1, v/v) for 24 hours. The degradation with thermolysin (5) performed at 37°C in 0.2 M NH_4HCO_3 solution at pH 7.8 for 48 hours in the presence of N-ethylmaleimide at a concentration of 10^{-3} M (15, 16). The acid protease digest was fractioned on a Sephadex G-25 column (2.2 x 240 cm) in 0.1 M formic acid. The peptides obtained were further purified by paper chromatography in n-butanol-acetic acid-water, 3:1:1, v/v, and by high voltage paper eletrophoresis in pyridine-acetic acid-water, 1:10:289, v/v, pH 3.6. Cystine peptides were developed with cyanide nitroprusside reagent (18). The thermolysin peptides were also purified by paper chromatography and paper electophoresis in the solvent systems described above. 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 the addition of a few drops of a solution of freshly prepared performic acid (1 volume of 30% H_2O_2 and 8 volumes of formic acid). After the oxidation had been allowed to proceed for 30 min., a few drops of water were added and the

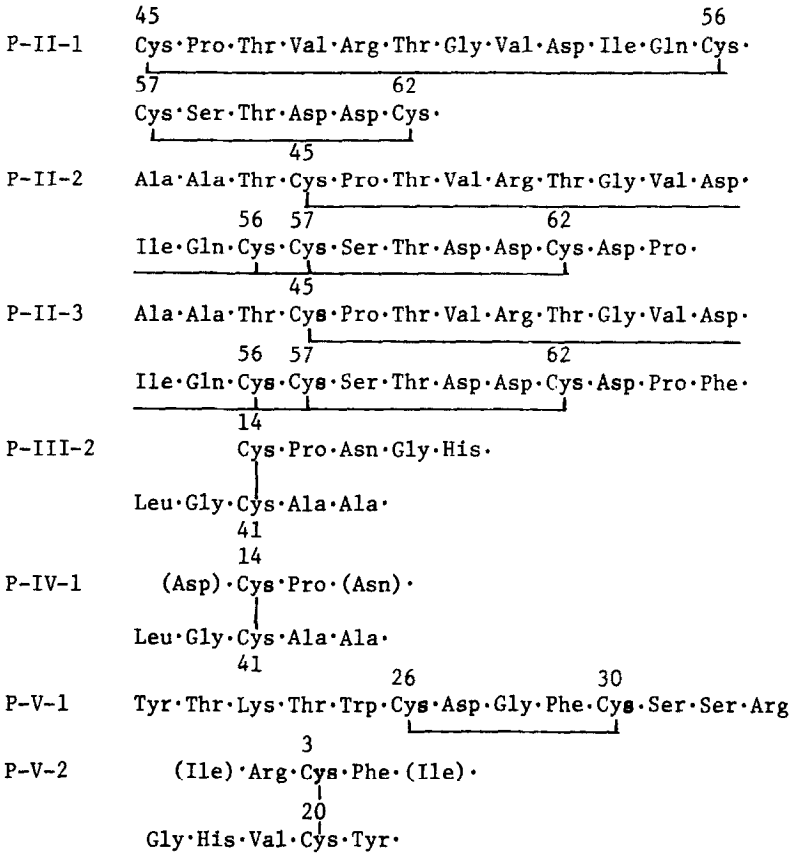

Fig. 2. Structures of the Peptides Obtained from Acid Protease Digest of Toxin B

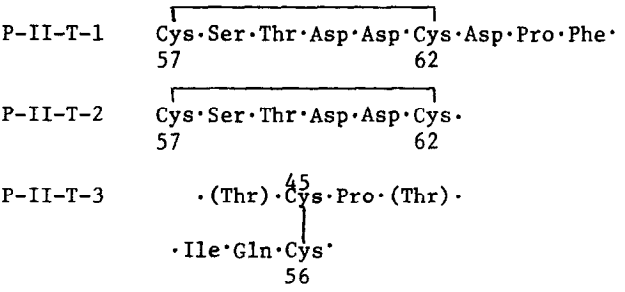

Fig. 3. Structures of the Thermolytic Peptides of Fraction P-II

solution taken to dryness. The residue was subjected to paper electrophoresis at pH 3.6 in order to separate the pure peptides.

RESULTS AND DISCUSSION

The disulfide bridges in some of the neurotoxins (5, 18, 19) and the

cytotoxin (20) isolated from the venom of Elapidae are located in corresponding positions. Despite the dissimilarity in their amino acid compositions and the number of disulfide bridges, the localization of at least eight cysteine residues in these cyto- and neurotoxins bear a distinct resemblance to each other. We considered the possibility that two cysteine residues out of ten might be linked across another disulfide bridge in the molecule and therefore analyzed carefully the cystine-containing peptides obtained by acid protease and thermolysin degradation. Toxin B was digested with acid protease and the digest dissolved in 0.1 M formic 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 226 and 280 nm (Fig. 1). The u. v. absorbing fractions P-I to P-V were collected and freeze-dried. Each fraction was subjected to paper electrophoresis at pH 3.6. The cystine containing-peptides were detected by cyanide nitroprusside spray. The areas occupied by cystine peptides were cut out and eluted with 10% acetic acid. The peptides so obtained were further purified by descending paper chromatography in n-butanol-acetic acid-water, 3:1:1, v/v. The peptides so obtained were homogeneous by the criteria of paper chromatography and electrophoresis. Each peptide was oxidized with performic acid, 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. From these results, the structures of the individual peptides could be formulated as shown in Fig. 2. After performic acid oxidation, peptide P-V-I consisted of only one component. Three of the five disulfide bridges were found between half-cystine residues 3 and 20, 14 and 41, and 26 and 30. As anticipated the remaining two disulfide bridges were both in one peptide in the P-II fractions. Positioning of the latter two disulfide bridges required cleavage of the peptide chain between the Cys-Cys sequence. The peptide bond Cys-Cys was

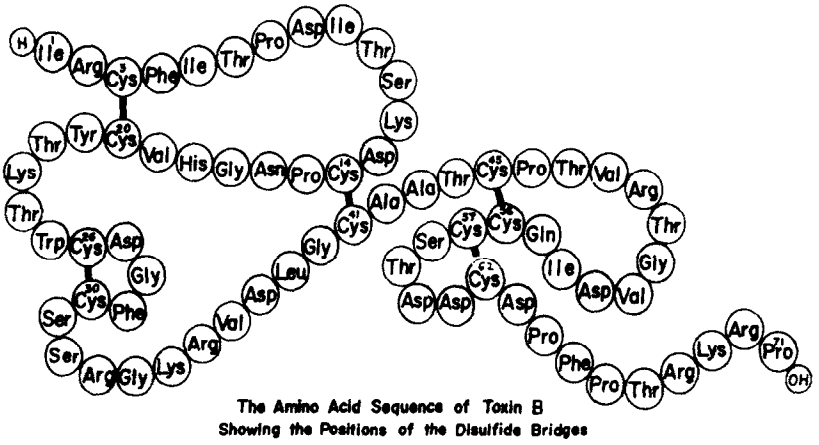

Fig. 4.

largely resistant to cleavage by trypsin, chymotrypsin, pronase and acid protease. Cleavage at the Cys-Cys bond took place only on prolonged treatment with thermolysin by which the equivlent Cys-Cys linkage of cytotoxin II was also successfully cleaved (20). The amino acid composition and the amino acid sequences of the peptides obtained from the thermolysin digest of P-II are shown in Table II and Fig. 3, respectively. From the structures of P-II-T-1, P-II-T-2 and P-II-T-3, it follows that the remaining disulfide bridges are between half cystine residues 45 and 56, and 57 and 62. The amino acid composition of the peptides presented in Tables I and II agree completely with the amino acid sequence of toxin B and the assumption of disulfide bridges between half cystine residues at positions 3 and 20, 14 and 41, 26 and 30, 45 and 56, and 57 and 62.

The complete structure of toxin B, is presented in Fig. 4. There is a remarkable similarilty between the position of the disulfide bridges of toxin B and those of the neurotoxins of known structure. The homologous half-cystine residues are in fact identically cross-linked in the neurotoxins, such as cobratoxin (19), erabutoxin (18) and cytotoxin II (20). The effect of the additional disulfide bridge on the toxicity of toxin B is under investigation.

Table I

Amino Acid Compositions of Disulfide Peptides and Their Oxidized Components (A) from Toxin B

	P-II-1	P-II-2	P-II-3	P-III-2			P-IV-1			P-V-1*		P-V-2		
				Total	A-1	A-2	Total	A-1	A-2	Total	A-1*	Total	A-1	A-2
Cysteic acid					1.00	1.00		1.00	1.00		2.00		1.00	1.00
Aspartic acid	2.82	3.60	3.78	1.20	1.12		1.00	0.88		1.00	1.13			
Threonine	2.77	3.65	3.40							1.77	1.87			
Serine	1.00	1.00	1.20							1.77	1.92			
Glutamic acid	0.80	0.94	1.00											
Proline	0.97	2.00	2.02	1.00	1.07		0.93	1.10						
Glycine	0.82	1.01	1.09	1.90	1.10	1.31	1.00		1.09	1.04	1.21	1.00		1.30
Alanine		1.56	1.56	1.83		2.18	1.87		1.86					
Half-cystine	3.01	3.17	4.30	1.95			1.74			1.66		1.77		
Valine	1.51	1.40	1.80									0.98		0.96
Methionine														
Isoleucine	0.75	0.76										1.00	1.00	
Leucine				0.70		0.88	0.79		0.77					
Tyrosine										0.77	0.80	0.87		0.85
Phenylalanine			0.72							1.20	0.95	1.13	0.95	
Lysine										0.82	1.00			
Histidine				0.70	0.70							1.00		0.68
Arginine	0.66	0.62	1.03							1.24	0.87	1.10	0.96	

The value of the amino acid underlined was taken as 1.0.
* Tryptophan was detected by Ehrich reaction.

Table II

Amino Acid Composition of Thermolytic Peptides of Fraction P-II and Oxidized Components (A) from P-II-T-3

Amino acid	P-II-T-1	P-II-T-2	P-II-T-3 Total	A-1	A-2
Cysteic acid				1.00	1.00
Aspartic acid	2.70	1.85			
Threonine	1.19	1.01	0.81	1.21	
Serine	0.95	1.00			
Glutamic acid			1.00		1.22
Proline	1.08		1.07	1.31	
Glycine					
Alanine					
Half-cystine	1.87	2.20	1.88		
Valine					
Methionine					
Isoleucine			0.78		0.91
Leucine					
Tyrosine					
Phenylalanine	1.00				
Lysine					
Histidine					
Arginine					

The value of the amino acid underlined was taken as 1.0.

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REFERENCES

1. Botes, D. P. and Strydom, D. J., J. Biol. Chem., 244, 4147 (1969).
2. Yang, C. C., Yang, H. J., and Husang, J. S., Biochem. Biophys. Acta, 188, 65 (1969).
3. Eaker, D. L. and Porath, J., Jap. J. Microbiol., 11, 353 (1967).
4. Botes, D. P., Strydom, D. J., Anderson, C. G., and Christensen, P. A., J. Biol. Chem., 246, 3132 (1971).
5. Botes, D. P., J. Biol. Chem., 246, 7383 (1971).
6. Mebs, D., Narita, K., Iwanaga, S., Samejima, Y. and Lee, C. Y., Hoppe-Seyler's Zeitsch. für Physiol. Chem., 353 243 (1972).
7. Nakai, K., Sasaki, T., and Hayashi, K. Biochem. Biophys. Res. Commun., 44, 893 (1971).
8. Botes, D. P., J. Biol. Chem., 247, 2866 (1972).
9. Strydom, A. J. C., and Botes, D. P., J. Biol. Chem., 246, 1341 (1971).
10. Sato, S., and Tamiya, N., Biochem. J., 122, 453 (1971).
11. Tamiya, N., and Abe, H., Biochem. J., 130, 547 (1972).

12. Strydom, D. J., J. Biol. Chem., 247, 4029 (1972).
13. Hayashi, K., et al., to be published.
14. Nakai, K. Nakai, C., Sasaki, T., Kakiuchi, K., and Hayashi, K., Naturwissenschaften, 57, 387 (1970).
15. Ryle, A. P., Sanger, F., Smith, L. F., and Kitai, R., Biochem. J., 60, 541 (1955).
16. Spackman, D. H., Stein, W. H., and Moore, S. J., J. Biol. Chem., 235, 648 (1960).
17. Toennies, G., and Kolb, J. J., Anal. Chem., 23, 823 (1951).
18. Endo, Y., Sato, S., Ishii, S., and Tamiya, N., Biochem. J., 122, 463 (1971).
19. Yang, C. C., Yang, H. J., Chiu, R. H. C., Biochem. Biophys. Acta, 214., 355 (1970).
20. Takechi, M., and Hayashi, K., Biochem. Biophys. Res. Commun., 49, 584 (1972).