CHEMICAL MODIFICATION OF THE TRYPTOPHAN RESIDUE IN TOXIN B FROM THE VENOM OF THE INDIAN COBRA*

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SUMMARY

The single tryptophan residue in toxin B has been converted into N'-formylkynurenine by ozonization in anhydrous formic acid, and also modified by reactions with 2-hydroxy-5-nitrobenzyl (HNB) bromide and 2-nitro-4-carboxyphenylsulphenyl (NCPS) chloride. Amino acid analyses of such modified derivatives show these reactions to be specific for tryptophan without significant effect to other amino acids. Ozonized toxin B has a residual toxicity of 80 %, and other tryptophan modified toxins retain at least half the toxicity of native toxin B. Each modified derivative gave a single fused precipitin line with native toxin on immunodiffusion against antitoxin B sera. In heterologous precipitin reactions, no significant decreases in antigenic activity of the modified derivatives were observed. The tryptophan residue at position 25 may, therefore, be part of neither the active site nor the antigenic site.

There are two types of neurotoxins in Elapidae and Hyrophiidae venoms. The neurotoxin type I consists of 60-62 amino acid residues, while the type II is composed of 71-74 amino acid residues (1). Earlier investigation of tryptophan residue in snake venoms were done on the type I neurotoxins. The chemical modification of tryptophan in the type II toxins has not yet been investigated. The sequence of 71 amino acids (2) and the position of the five disulfide bridges (3) in toxin B has been determined. Selective nitration of the single tyrosine residue (Tyr-21) in toxin B did not affect toxicity (4). In this respect, as explained in the discussion, the type II neurotoxins differ markedly from the type I neurotoxins. Likewise, toxin B, in analogy to most other neurotoxins, contains one single tryptophan (Trp-25). Chemical modification of the analogous Trp-28 in neurotoxins isolated from the venom of sea snakes and cobras has shown its importance for toxicity (5, 6, 7, 8, 9). In this study, Trp-25 of toxin B has been modified by three different procedures, viz., selective ozonization in anhydrous formic acid to N'-formylkynurenine without breakage of the peptide chain (10, 11), specific alkylation by HNB bromide (12, 13) and NCPS chloride (14). The

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modified toxins have then been evaluated on the basis of their antigenic specificities.

MATERIALS AND METHODS

Toxin B was prepared from the venom of the Indian cobra as previously reported (15). Reagent grade HNB bromide was purchased from Seikagaku Kogyo Co., Ltd., Japan, and NCPS chloride was a gift from Dr. M. Ohno of Kyushu University.

Ozonization of Toxin B. Ozonization was carried out according to the published procedure (16). Toxin B was dissolved in anhydrous formic acid (10 mg/ml) and treated with a slow stream of ozone (2.07 μmoles per min) at 8 °C. At suitable intervals, 0.1 ml aliquots were withdrawn from the reaction mixture and transferred into 3 ml of water for spectrophotometric determinations, then lyophilized and used for toxicity assays and Ouchterlony tests.

Modification with HNB Bromide. Alkylation with HNB bromide was carried out under standard conditions (12, 13). Two μmoles of toxin B were preincubated for 20 hours at 37 °C in 1 ml of 10 M urea which had been adjusted to pH 2.7 with concentrated HCl. After incubation, a 2-, 10-, or 50-fold molar excess of HNB bromide in 0.2 ml of dry acetone was added, respectively, with a pipette below the surface of the protein solution with vigorous agitation. After reaction of 1 hour at room temperature, the modified toxin was separated on a column of Sephadex G-25 in 1% acetic acid as a solvent and the protein fractions were pooled and lyophilized.

Modification with NCPS Chloride. Sulphenylation was carried out by the method of Scoffone et al. (14). To a solution of toxin (2 μmoles) in 2 ml of 30% acetic acid, a 10-fold molar excess of NCPS chloride in 0.5 ml of glacial acetic acid was added. The reaction was allowed to proceed at room temperature for 10 min and the mixture was desalted by passage through a Sephadex G-25 column equilibrated with 0.2% acetic acid. The protein fractions were pooled and lyophilized.

Amino Acid Analysis. About 1 mg of protein was hydrolyzed in 1 ml of 6 N HCl in an evacuated sealed tube for 24 hours at 105 °C. The hydrolyzate was analyzed on a Hitachi Model KLA-3B automatic amino acid analyzer.

Measurement of Toxicity. The toxicity of toxin B and modified toxin was measured by intraperitoneal injection (mice, 16-18 g) of a progressively diluted toxin solution. Toxin B has an LD_{50} of 0.15 μg/g in mice. Four mice of both sexes were used for each dilution and the LD_{50} was calculated according to the 50 % end point method of Reed and Muench (17).

Preparation of Antitoxin B Sera. Antitoxin B sera were prepared in

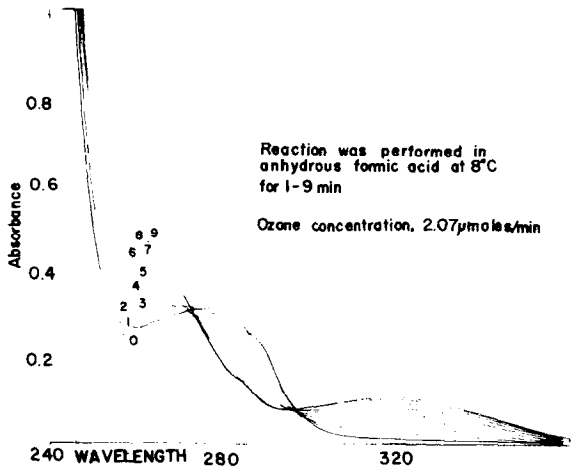

Fig. 1. Absorption spectra of ozonized-toxin B.

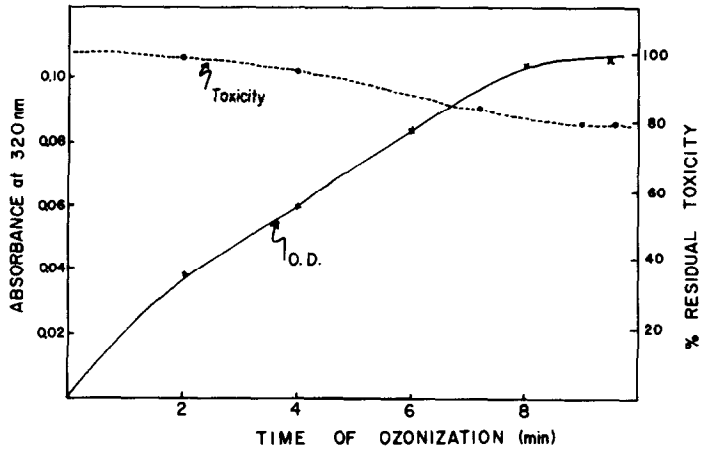

Fig. 2. Relationships between the formation of N'-formyl-kynurenine and the toxicity of toxin B as a function of time of ozonization.

rabbits weighing 2.0-2.5 kg by injecting increasing amounts of the toxin B with complete Freund's adjuvant. Doses varying from 10 μg to 1.5 mg of toxin B per kg body weight were injected subcutaneously into the right and left thigh alternately at weekly intervals during a period of three months and the animals bled 10 days after the final injection.

Immunological Procedures. Double diffusion in agar gel was performed by Ouchterlony's technique (18). The quantitative precipitin reactions were carried out as described by Kabat and Mayer (19). Increasing amounts of

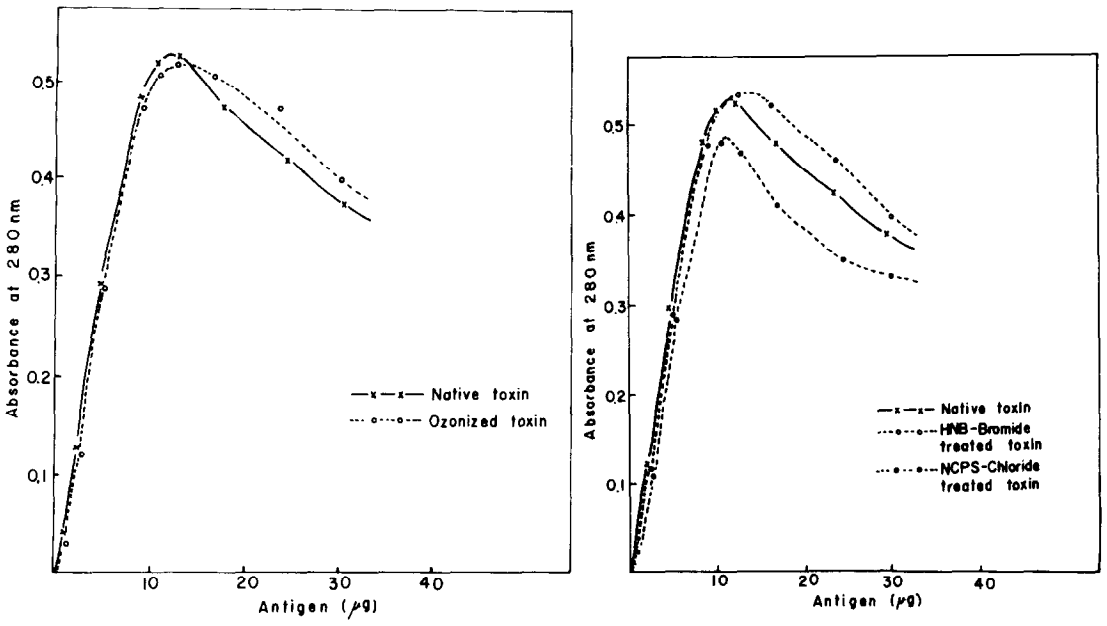

Fig. 3. Comparison of the precipitin curves of toxin B and chemically modified derivatives with anti-toxin B sera.

antigen in phosphate-buffered saline were added to a constant amount of anti-serum in a total volume of 0.15 ml. The tubes were incubated for 30 min at 37 °C and then left overnight at 4 °C. The precipitates were washed 3 times with cold phosphate-buffered saline and then measured at 280 nm.

RESULTS AND DISCUSSION

The extent of ozonization was followed by measuring the increasing absorbance at 320 nm of N'-formylkynurenine (10, 11). As shown in Fig. 1, the absorbancy at max 280 nm decreased, while the two peaks at 260 and 320 nm corresponding to the two maxima of N'-formylkynurenine increased. The absorbance increment at 320 nm indicated the extent of tryptophan modification as a function of time. The relationship between toxicity and the formation of N'-formylkynurenine is shown in Fig. 2. Although, commensurate with the N'-formylkynurenine formed, toxicity was slightly decreased, residual toxicity of toxin B remained stable after 9 min at 80 %. Control experiments without ozone confirmed that toxicity was not affected under these conditions. The venom is stable to acid and to short-term ozonization. After ozonization for 9 min, as shown in Table I, one tryptophan per mole of toxin B was converted to N'-formylkynurenine, while all other amino acids remained intact, except for a small amount of cysteic acid formed from some of the 10 half-cystine residues, an observation which may explain the slight drop in toxicity.

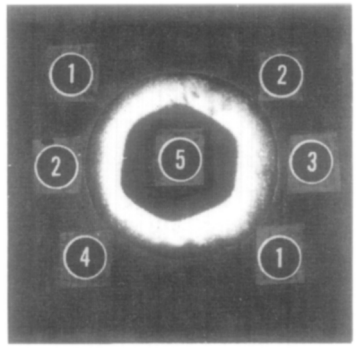

Fig. 4. Immunodiffusion in agar gel. Central well, antitoxin B sera. Surrounding wells: (1) toxin B; (2) ozonized toxin B; (3) HNB-toxin B; (4) NCPS-toxin B.

The reactions of toxin B with HNB bromide or NCPS chloride, were monitored spectrophotometrically on the basis of molar absorbancy coefficients of 18,900 at 410 nm and of 4,000 at 365 nm, respectively. In this way lyophilized samples after modification with HNB bromide, dissolved in 0.1 N sodium acetate-NaOH buffer, pH 12.7, showed that 0.11, 0.68 and 0.95 moles of tryptophan per mole of toxin B had been modified by reaction with a 2-, 10- and 50-fold molar excess of HNB bromide residual toxicity was 50%. Sulphenylation of tryptophan followed spectrophotometrically in 0.2 M acetic acid solution demonstrated a loss of toxicity of 54% when 1.05 moles of NCPS-tryptophan had been formed. Amino acid analyses of the modified derivatives confirmed that, apart from tryptophan, all other amino acids remained intact (Table I).

As shown in Fig. 3 the antigenic activity of the modified derivatives did not significantly decrease in the heterologous precipitin reactions with antitoxin B sera. However, precipitation was slightly decreased with the NCPS chloride-treated derivative. Also, these modified derivatives gave a precipitin line identical with toxin B as tested by immunodiffusion in agar gel with antitoxin B serum (Fig. 4).

The chemical modification of the tryptophan residue (Trp-28) has been carried out on cobratoxin obtained from the venom of naja naja atra (8). Also, another chemical agent such as N-bromosuccimide was used for tryptophan modification with different snake toxins and the modified toxins were detoxified (5, 6, 7, 9, 20). For that reason, Trp-28 was considered to be one of the essential components of the active site. On the other hand, when Chicheportiche et al. (21) noticed that the N-formylated neurotoxin I from naja haje has a residual toxicity of about 50% and no activity is regained after deformylation, he concluded: "It is probably more reasonable to think

Table I

Amino Acid Composition of Toxin B Derivatives

	Residues/mole of protein			
Amino acids	Native Toxin B	Ozonized toxin B*	HNB bromide-treated toxin	NCPS chloride-treated toxin
Cysteic acid	-	0.43	-	-
Aspartic acid	9	8.95	8.80	8.71
Treonine	9	8.83	8.16	8.32
Serine	4	4.06	3.66	3.46
Glutamic acid	1	1.14	1.08	1.20
Proline	6	6.05	5.91	5.54
Glycine	5	4.78	5.08	5.13
Alanine	2	2.15	2.23	1.90
Half-cystine	10	8.83	8.95	8.35
Valine	4	3.31	3.48	3.36
Methionine	0	0	0	0
Isoleucine	4	3.40	3.91	3.81
Leucine	1	1.00**	1.00	1.00
Tyrosine	1	1.04	0.96	0.86
Phenylalanine	3	2.78	2.86	2.76
Lysine	4	3.84	3.80	3.52
Histidine	1	0.86	0.78	0.91
Arginine	6	5.36	5.10	5.21
Tryptophan	1	-	-	-
Kynurenine	-	0.93	-	-
HNB-tryptophan	-	-	0.95***	-
NCPS-tryptophan	-	-	-	1.01***

* Sample treated with ozone for 9 min.
** All values are expressed as molar ratios with a value of 1.0 for leucine as standard.
*** Determined spectrophotometrically.

that the side chain of Trp-28, like the phenol part of tyrosine-24, is only involved in the stabilization of an adequate geometry for the active site (21)."

Our own studies on ozonization, alkylation and immunology of toxin B suggest that Trp-25 is neither part of the active site nor the antigenic site itself. The marked differences between the toxins of type I and type II may by the result of the additional disulfide bridge between cysteine residues 26 and 30 in toxin B.

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