CHEMICAL MODIFICATION OF THE TRYPTOPHAN RESIDUE IN COBRATOXIN*

C. C. Chang** and K. Hayashi***

Department of Biological Chemistry, Faculty of Pharmaceutical Sciences
Kyoto University, Kyoto, Japan

Received October 7, 1969

SUMMARY

The single tryptophan residue in cobratoxin has been converted into N'-formyl-kynurenine by ozonization in formic acid, and also modified by reaction with 2-hydroxy-5-nitrobenzyl bromide. The data from amino acid analysis of modified derivatives show that these reactions are specific for tryptophan, and do not affect the other amino acids. The low toxicity of these modified products points to the importance of the intact tryptophan residue in cobratoxin for full activity.

Cobratoxin, the crystalline toxic protein obtained from Formosan cobra venom by Yang (1), consists of a single polypeptide chain of 62 amino acids which are cross-linked by four disulfide bonds (2). The complete amino acid sequence which has recently been determined (3) permits a study of structure-activity relationships.

There is in cobratoxin one single tryptophan located at position 29. There are no methionine, alanine, phenylalanine, and free cysteine residues (2-4). Tryptophan, which may be modified by a variety of chemical procedures, is selectively ozonized in anhydrous formic acid to N'-formyl-kynurenine without breakage of the peptide chain (5,6) and is alkylated by 2-hydroxy-5-nitrobenzyl bromide (7,8) in the 3-position (9) with a high degree of specificity.

We now report on the modification of tryptophan in cobratoxin by these two procedures and on the effects of these reactions on the activity of the venom.

*Supported in part by the National Science Council, Republic of China.

**Present address: Department of Biochemistry, Kaohsiung Medical College, Kaohsiung, Taiwan, Republic of China.

***To whom inquiries about this article should be directed.

MATERIALS AND METHODS

Cobratoxin was prepared from Formosan cobra (Naja naja atra) venom as previously described (1).

Ozonization of Cobratoxin: Ozonization was carried out according to the procedure as described (10). Cobratoxin was dissolved in anhydrous formic acid (10 mg/ml) and treated with a slow stream of ozone at 8°. At suitable intervals, 0.1 ml aliquots were withdrawn from the reaction mixture and transferred into 3 ml of H_2O for spectrophotometric determinations, then lyophilized and used for toxicity assays.

Modification with 2-Hydroxy-5-nitrobenzyl Bromide (HNB Bromide): The alkylation with HNB bromide was carried out under standard conditions (8). Fifty mg of cobratoxin was pre-incubated for 20 hr at 37° in 3 ml of 10 M urea which had been adjusted to pH 2.7 with concentrated HCl. After incubation, a 10-fold molar excess of HNB bromide in 0.3 ml of dry acetone was added with a pipette below the surface of the protein solution with vigorous agitation. After reaction for 1 hr at room temperature the modified toxin was separated from excess reagent on a column (2.8 x 55 cm) of Sephadex G-25 equilibrated with 0.18 M acetic acid (pH 2.7) and the protein fractions were pooled and lyophilized.

Amino Acid Analysis: About 0.2 μmole of protein sample was hydrolyzed in 1.0-1.5 ml of 6 N HCl in an evacuated, sealed tube for 24 hr at 105°. The hydrolysate was analyzed (11) on a Beckman Spinco Model MS or a Hitachi Model KLA-3B automatic amino acid analyzer.

Measurement of Toxicity: The activity of the venom was measured by intraperitoneal injection (mice, 16-18 g) of a progressively diluted venom solution as described previously (12). 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 (13).

RESULTS AND DISCUSSION

Ozonization: The ozonization of tryptophan to N'-formyl-kynurenine was

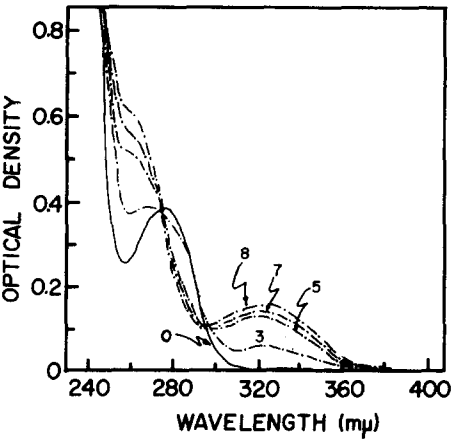

Fig. 1. Absorption spectra of cobratoxin before and after ozonization. Each sample (1 mg in 0.1 ml of anhydrous formic acid) was transferred into 3 ml of H_2O and the spectra were recorded with a Beckman DB spectrophotometer. The numbers in the figure denote the time of ozonization in minutes.

followed by the changes in uv absorption (Fig. 1). The absorbancy at λ_{max} 280 mμ decreased, while two peaks at 260 and 320 mμ corresponding to the two maxima of N'-formyl-kynurenine (14) increased. The O.D. increment at 320 mμ corresponding to the formation of N'-formyl-kynurenine indicated the extent of tryptophan modification as a function of time.

The relationship between toxicity and the formation of N'-formyl-kynurenine is shown in Fig. 2. With increasing amounts of N'-formyl-kynurenine, toxicity

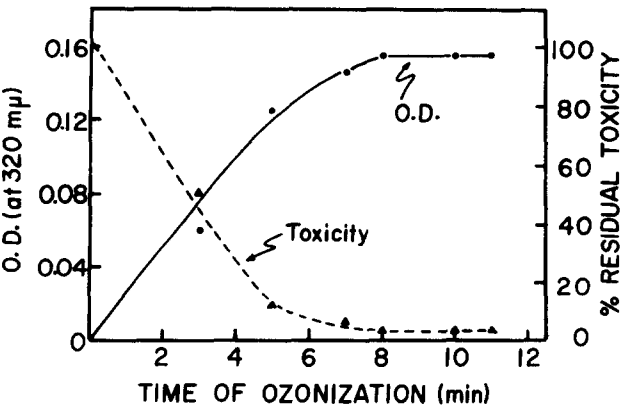

Fig. 2. Relationships between the formation of N'-formyl-kynurenine and the toxicity of cobratoxin as a function of time of ozonization.

TABLE I

Toxicity of Cobratoxin Derivatives

Cobratoxin derivatives	Toxicity in %
Native cobratoxin	100.0 (0.069)*
Ozonized cobratoxin**	3.1 (2.212)
HNB bromide-treated cobratoxin	6.2 (1.106)

*The numbers in parentheses denote the LD_{50}, mg/kg body weight of mouse, and was measured by intraperitoneal injection of the venom solution into mice, weighing 16-18 g as previously described (12).

**Sample treated with ozone for 8 min.

decreased progressively and finally was down to 3.1% of native cobratoxin after ozonization for 8 min (Table I). No further changes in the absorbancy at 320 mμ and the toxicity were observed after 8 min. Control experiment without ozone confirmed that the toxicity of the venom was not affected under these conditions. This is accounted for by the stability of the venom to acid and the short duration of the ozonization.

Amino acids were analyzed on lyophilized samples after ozonization for 8 min. As shown in Table II essentially one tryptophan per mole of cobratoxin was converted to N'-formyl-kynurenine, while there were no significant changes in the contents of the other amino acids.

Reaction with HNB Bromide: On electrophoresis the modified sample, λ_{max} 410 mμ, gave a single band migrating more slowly toward the anode relative to native venom. No unreacted venom was detected by polyacrylamide gel electrophoresis. Spectral determination of the extent of modification based on a molar extinction coefficient of 18,900 from O.D. at 410 mμ of lyophilized sample dissolved in 0.1 N sodium acetate-NaOH buffer, pH 12.7 (15) suggested that >0.92 mole of tryptophan per mole of cobratoxin had reacted with HNB bromide resulting in a loss of toxicity of about 94% (Table I). The amino

TABLE II

Amino Acid Composition of Cobratoxin Derivatives

Amino acids	Residues/mole of protein		
	Native cobratoxin	Ozonized cobratoxin*	HNB bromide-treated cobratoxin
$CysSO_3H$	-	0.34	0
Asp	8	8.4	8.2
Thr	8	7.5	7.9
Ser	4	3.3	3.9
Glu	7	7.1	7.3
Pro	2	2.0	2.3
Gly	7	7.2	7.3
Ala	-	-	-
Cys(1/2)	8	8.3	7.9
Val	1	1.0	1.1
Met	-	-	-
Ile	2	2.0	2.1
Leu	1	1.0**	1.0
Tyr	2	1.7	2.1
Phe	-	-	-
Lys	3	3.0	3.1
His	2	1.7	1.8
Arg	6	6.1	6.3
Trp	1	0.0	0.0
Kynurenine	-	0.75	-
HNB-Trp	-	-	0.92***

*Sample treated with ozone for 8 min.

**All values are expressed as molar ratios with a value of 1.0 for leucine as standard.

***Determined spectrophotometrically at 410 mμ.

acid analysis showed that, besides tryptophan, all other amino acids remained intact (Table II).

It still remains to be seen whether other modifications of tryptophan, e.g. by N-bromosuccinimide or tetranitromethane, are possible without loss of activity as is the case in lysozyme (16).

ACKNOWLEDGMENTS

The authors are very much indebted to Dr. B. Witkop of N. I. H. for his help and comments in preparing this manuscript, and also to Professor I. Yamashima of Kyoto University and Professor C. C. Yang, Dean of Kaohsiung Medical College

for his encouragement during the course of the study. Thanks are also due to Dr. F. Sakiyama for assistance with the ozonization and amino acid analyses, and Mr. K. Nakai for the technical assistance.

REFERENCES

1. Yang, C. C., J. Biol. Chem. 240, 1616 (1965).
2. Yang, C. C., Chang, C. C., Hayashi, K., and Suzuki, T., Toxicon 7, 43 (1969).
3. Yang, C. C., Yang, H. G., and Huang, J. S., Biochim. Biophys. Acta 188, 65 (1969).
4. Yang, C. C., Biochim. Biophys. Acta 133, 346 (1967).
5. Witkop, B., Ann. 556, 103 (1944).
6. Previero, A., and Bordingnon, E., Gazz. Chim. Ital. 94, 630 (1964).
7. Horton, H. R., and Koshland, Jr., D. E., J. Amer. Chem. Soc. 87, 1126 (1965).
8. Barman, T. E., and Koshland, Jr., D. E., J. Biol. Chem. 242, 5771 (1967).
9. Spande, T. F., Wilchek, M., and Witkop, B., J. Amer. Chem. Soc. 90, 3256 (1968).
10. Previero, A., Colett-Previero, M-A., and Jollès, P., J. Mol. Biol. 24, 261 (1967).
11. Spackman, D. H., Stein, W. H., and Moore, S., Anal. Chem. 30, 1190 (1958).
12. Yang, C. C., J. Formosan Med. Assoc., 63, 325 (1964).
13. Reed, L. J., and Muench, H., Am. J. Hyg. 27, 493 (1938).
14. Dalgliesh, C. E., J. Chem. Soc. 137 (1952).
15. Koshland, Jr., D. E., Karkhanis, Y. D., and Latham, H. G., J. Amer. Chem. Soc. 86, 1448 (1964).
16. Cf. Witkop, B., Science 162, 318 (1968).