Toxicity of the Surgeonfishes—II Properties of the Principal Water-soluble Toxin

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Specimens of surgeonfish *Ctenochaetus striatus* collected in Tahiti contained a water-soluble toxin in addition to ciguatoxin. The water-soluble toxin was tentatively named maitotoxin (MT) after the Tahitian name for surgeonfish maito. Purification of MT was achievable by the standard purification procedures for polar lipids. The toxin was eluted from a silicic acid column with chloroform-methanol (6:4) and from a cellulose column with chloroform-methanol-water (5:15:1). Upon gel-filtration through Sephadex G-25, it appeared in the fractions near void volume. Acid hydrolysis of the toxin afforded fatty acids, glucose and galactose, and 15 amino acids. The minimum lethal dose to mice by ip injection was estimated to be 15~20 mg/kg. Guppies put in 40 ppm solution of MT died within 150 min. These chemical and physiological properties indicated a close similarity of MT to the ichthyotoxin produced by the phytoflagellate *Prymnesium parvum*.

The term ciguatera has been applied to a variety of symptoms resulting from ingestion of fresh coral reef fish. Many biological and ecological studies¹⁻³⁾ suggested that the principal toxin, isolated from the fat soluble fraction of canivorous fish and named ciguatoxin,⁴⁾ might be produced by a benthic microorganism and transmitted by herbivorous fish to the carnivores through food chain. This prediction was confirmed in our previous study⁵⁾ by the recovery of ciguatoxin from the stomach contents of a herbivorous surgeonfish *Ctenochaetus striatus*. In addition to ciguatoxin the presence of other toxins was also recognized at the same time. Since the illness labelled as ciguatera shows a wide range of variability in symptoms,⁶⁾ it seems necessary to have a more definite knowledge of the secondary toxins responsible for this phenomenon. Among such secondary toxins a component mentioned as the water-soluble toxin in the previous report was judged to be the most important one. In the present paper this toxin is designated as maitotoxin (MT) after the Tahitian name maito for *C. striatus* to be distinguished from other secondary toxins.

The present paper deals with the purification and chemical properties of MT and includes a discussion on the similarity of MT to *Prymnesium parvum* toxins. A supplemental data on the chromatographic behavior of other minor toxins are also presented.

Materials and Methods

Specimens Purification of MT was carried out with the partially purified sample

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obtained in the previous study.⁶⁾ The sample had been extracted from the specimens of *C. striatus* collected at Popote Bay, Tahiti, in 1968 and 1969. Additional small batches of specimens were also collected at Tahiti in 1974. The minimum lethal dose of the sample to mice by intraperitoneal (ip) injection was 140 mg/kg. Its hemolytic activity corresponded to 80% that of the standard saponin (Merck, Darmstadt). The guppies put in a 44 ppm solution died in 26~34 min.

Toxicity test Toxicity was assayed by ip injection in female mice weighing about 20 g each. For testing the toxicity by oral administration, 10 mg of MT dissolved in 30 ml of milk was fed respectively to two cats weighing 370 and 400 g. The hemolytic activity was measured by the method used for asterosaponin.⁷⁾ Ichthyotoxicity was tested by putting five guppies *Lebistes reticulatus* in a beaker maintained at 25°C and containing 50 ml of 40 ppm solution of MT.

Purification of toxins by chromatography The sample of MT (920 mg, 140 mg/kg) was fractionated first on a silicic acid column with chloroform containing methanol in various ratios and each fraction was tested for toxicity to mice and hemolytic activity. The toxic eluate from the silicic acid column was then subjected to gel-filtration through a Sephadex G-25 column. The toxin was chromatographed once again on a silicic acid column with 1-propanol containing 20% water. Since glycolipid-like nature of MT was suggested by qualitative test, two additional steps of chromatography were carried out to see if the carbohydrate contents in the eluates were in parallel with toxicity. First, MT was chromatographed over a silicic acid column with a solvent consisting of 1-butanol, ethanol, and water (4: 1: 2). Secondly, cellulose column chromatography was carried out after the manner of WOLFE and LOWDEN.⁸⁾ The eluate was monitored for both toxicity to mice and carbohydrate content with anthrone-sulfuric acid reagent.

For thin layer chromatography Wako gel B-O was used as the adsorbent. Three solvent systems were used: (A) 1-propanol-water (7: 3), (B) 1-butanol-ethanol-water (4: 1: 2), and (C) chloroform-methanol-water (60: 35: 8). Detection of the spot was made by anthrone-sulfuric acid reagent.

Other secondary toxins obtained from the fat-soluble fraction were first treated on silicic acid columns with chloroform containing methanol and next on an acidic alumina column with the similar solvent system.

Identification of hydrolysis products Analyses of fatty acids, carbohydrates and sphingosine were carried out after the method of SWEELY and his group.^{9,10)} By methanolysis of MT with dilute methanolic hydrochloric acid, the fatty acid portion was converted to methyl esters and the oligosaccharide portion to monomeric carbohydrate. The carbohydrate and sphingosine were converted to trimethylsilyl derivatives and analyzed on an OV-17 column by gas chromatography. The fatty acid components were determined on a diethyleneglycol succinate column. A portion of the methanolysis products was

subjected to acetylation prior to trimethylsilylation in order to check the presence of hexosamine. For amino acid analysis, 1.5 mg of MT was hydrolyzed in 6 N hydrochloric acid at 110°C for 24 hr and the hydrolyzate was analyzed by a Hitachi KLA-2 automatic analyzer.

Results

The first fractionation on a silicic acid column revealed that the hemolytic activity of the crude MT preparation was independent of MT. Toxicity test with both mice and guppies indicated that MT was eluted from the column in chloroform-methanol (6:4) fraction, while the hemolytic component was eluted with methanol and was shown to be indistinguishable from lysophosphatidyl choline by thin layer chromatography. In gel-filtration MT appeared in the fractions near the void volume. Chromatography over a silicic acid column with 1-propanol-water (8:2) was effective in removing colored contaminants but the toxicity was not raised remarkably. The yield and toxicity of sample at each stage of purification are shown in Fig. 1. The results of

Fig. 1. Procedures for purification of MT.

Fig. 2

Fig. 2. Silicic acid column chromatography of partially purified MT with the solvent consisting of 1-butanol, ethanol, and water (4:1:1).

Fig. 3

Fig. 3. Cellulose column chromatography of partially purified MT.

silicic acid chromatography with 1-butanol-ethanol-water and cellulose column chromatography are shown in Figs. 2 and 3. It may be seen that the carbohydrate concentration of the eluate parallels well with the toxicity to mice. The minor toxic fraction recognized in the latter chromatography was not pursued further owing to its insufficient sample size.

The final preparation thus obtained was a slightly yellowish amorphous powder which killed mice at a dose between 15 and 20 mg/kg. The signs observed were a remarkable loss of activity, weakness in limbs, and convulsion before death which took place after 3 hr from injection or 3 days at the latest depending upon the doses. Guppies put in the 40 ppm solution of the toxin died in 150 min. Feeding of MT to cats induced within 30 min severe vomiting which lasted for nearly one hr. No other ill effect was observed. MT formed foamy emulsion in distilled water or dilute acetic acid solution but gave a clear solution in a dilute ammonium hydroxide solution. Treatment of MT with acidic media resulted in a remarkable loss of toxicity. Gradual decrease of toxicity was also recognized during the storage of dried MT preparation in a freezer at -20°C . On spot tests positive result was obtained with anthrone-sulfuric acid reagent but not with Dragendorff, ammonium molybdate, and aniline hydrogen phthalate reagents. An ambiguous result was obtained with ninhydrin reagent. Initially the toxin preparation was negative to the reagent but became positive after storage for several weeks. Thin layer chromatography indicated that the final preparation of MT was still not homogeneous but a mixture of at least two components: R_f 0.72 and 0.78 with solvent A, 0.32 and 0.35 with solvent B, and 0.17 and 0.30 with solvent C. Further attempts to separate the two components were unsuccessful. The IR spectrum of MT is shown in Fig. 4. The characteristic bands (cm^{-1}) in the spectrum may be assignable to the following groups: 3320 and 1080 (hydroxyl), 2900 and 2830 (methyl and methylene), 1640 and 1530 (amido), 970 (trans double bond), and 890 (glycosidic linkage). Absence of intense band near 1720 (ester) and 1240 (phosphatides or sulfatides) excludes possibility of the toxin being glycerophospholipids, sphingophospholipids, or sulfatides.

Gas chromatographic analysis of methanolysis products indicated the presence of

Fig. 4. IR spectrum of MT (KBr).

Table 1. Amino acid residues in hydrolyzate from 1.5 mg of MT

Amino acid	μ mole	μ g	Mole %
Asp	0.212	28.216	6.20
Thr	0.164	19.536	4.79
Ser	0.180	18.916	5.26
Glu	0.204	30.016	5.96
Pro	0.400	46.052	11.69
Gly	0.292	21.920	8.54
Ala	0.208	18.532	6.08
Val	0.216	25.304	6.31
Ile	0.164	21.512	4.79
Leu	0.276	36.204	8.07
Tyr	0.052	9.424	1.52
Phe	0.124	20.484	3.62
Lys	0.076	11.112	2.22
His	0.034	5.276	0.99
Arg	0.072	12.544	2.10
Ammonia	0.747	12.580	21.84
Total	3.421	337.628	99.98

glucose, galactose, and sphingosine-like base and the absence of hexosamine and neuraminic acid. The fatty acid portion consisted of two major components, palmitic acid (30%) and stearic acid (60%).

Result of amino acid analysis is presented in Table 1. At least 15 amino acids were

Fig. 5. The scheme for fractionation of the secondary toxins in the fat-soluble fraction with the yield of the residue (mg), minimum lethal dose (mg/kg) and death time (hr or min) at each stage.

recognizable in the hydrolyzate. The amino acid portion comprised about 24% of the final preparation.

As mentioned in the previous paper, occurrence of different toxins in a few batches of specimens was observed. A typical example of chromatographic behavior of such toxins is illustrated in Fig. 5. These minor toxins were distinguishable from ciguatoxin by the fast-death in mice.

Discussion

The present investigation proved that MT could be effectively purified by the standard procedures used for polar lipid purification. The constituents detected after degradation of the final preparation were fatty acids, sphingosine-like base, hexoses, and amino acids. Other polar lipids containing such constituents as phosphate, sulfate, neuraminic acid, and hexoamine disappeared as the purification proceeded. The concentration of sphingosine-like base in the hydrolyzate also decreased remarkably but it was still detectable in the final preparation. Since occurrence of sphingosine in bacteria is rather unusual except in those belonging to genus *Bacteroides*, its existence may be important in forecasting whether MT is a bacterial product or not. We are intending to carry out an experiment upon the presence of sphingosine with the more purified sample in future.

With regard to the origin of MT we have suggested previously that a benthic alga of fine structure, yeast, or bacterium might be the toxin elaborator. So far, several types of glycolipids possessing various biological activities have been known among the bacterial products. However, most of these bacterial glycolipids contain such constituents as phosphate, hexosamine, and neuraminic acid and, therefore, are not likely to be related to MT. As to the algae, occurrence of water-soluble toxins in the non-dialyzable fractions of various species has been reported by HASHIMOTO and FUSEYANI.¹¹⁾ However, lack of the knowledge of their chromatographic behaviors and chemical properties makes it impossible to compare these toxins with MT. On the other hand, a close similarity of MT to toxins of *Prymnesium parvum* is pointed out. This phytoflagellate is a member of Chrysophyta which consists of unicellular, colonial or filamentous, algae with or without flagella, and has been known to be responsible for high fish mortality in Europe and Israel. The isolation procedure employed by ULITZUR and SHILO¹²⁾ for the toxic principles indicates many parallels between their toxins and MT in the solubility in various organic solvents and water, as well as in the biphasic mixture. The chromatographic behaviors of the flagellate toxins on a silicic acid column and Sephadex column are also compatible with those of MT. Both toxins are sensitive to acids. A more direct basis for comparison is offered by the chemical analyses. The flagellate toxins were found to contain 20.4% protein or peptide consisting of 15 amino acids, a number of fatty acids, 0.4% phosphate, and 10~12% glucose and galactose. These data as well as the biological activity indicate

a close similarity of MT to *P. parvum* toxins.

So far, no evidence is obtained which connect MT with ciguatoxin both chemically and pharmacologically. The feeding experiment with cats indicated that ingestion of MT may induce some illness, probably vomiting and nausea. Future study on pharmacological activities will be necessary to elucidate the role of MT in ciguatera as well as other minor toxins found in the surgeonfish.

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