Diethyl Peroxides Probably Responsible for *Mozuku* Poisoning

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Brown marine algae of the families Chordariaceae and Nemacystaceae have sporadically caused human intoxications known as *mozuku* poisoning. In the present study, some of these seaweeds were extracted and tested for toxicity in mice. The fat soluble fractions prepared from *Sphaerotrichia divaricata* and *Cladosiphon okamuranus* were found to be toxic. Three toxic peroxides have been isolated from the former alga by using mainly chromatographic techniques. The principal toxin, α,α' -dihydroxy diethyl peroxide, has been synthesized to confirm its structure and toxicity. It was suggested that these toxins are most likely responsible for *mozuku* poisoning.

These algae seem to become toxic at a particular stage of their life cycle. It was also demonstrated that the toxins were quite labile in an algal body and were generated only when the body was heated with water. Similar peroxides have been found in the brown alga *Analipus japonicus* and also in the red alga *Gracilariopsis chorda*.

Brown seaweeds of the families Chordariaceae and Nemacystaceae are widely consumed as a side dish in Japan. However, it has long been believed in the Amami Islands as well as along the coast of Japan Sea that they might become toxic at late stages of life cycle and cause human intoxications known as *mozuku* poisoning¹⁾. In spite of this popular consumption only two cases of food poisoning due to the ingestion of these algae have been reported so far: the first incidence was caused by *Nemacystus decipiens* (Japanese name, 'mozuku') in Oga-shi, Akita, 1967 and the second case by *Cladosiphon okamuranus* ('okinawa-mozuku') on Yoron Island of the Amami group, 1974.

Toxicity of a few edible seaweeds has been reported^{1,2)}, but never been experimentally confirmed to be responsible for food poisoning. In 1975 being encouraged by the detailed case history of the Yoron incidence given by Professor K. Nozawa of Kagoshima University we started our work on *mozuku* poisoning. Fortunately, lethality in mice was detected in *Sphaerotrichia divaricata* ('ishi-mozuku') collected in Oga-shi, from which toxic peroxides have been isolated. These peroxides were believed to be responsible for the poisoning. The similar compounds were also found in *C. okamuranus*, *Analipus japonicus* ('matsumo') and even in a red alga *Gracilariopsis chorda* ('tsuru-shiramo').

This paper deals with these results.

Materials and Methods

Algal Specimens

All algal specimens were frozen immediately after collection, transported frozen to our laboratory, and kept in a freezer at -20°C . Samples of *C. okamuranus* were collected on Yoron Island, late June of 1976, *A. japonicus* at Sanriku-cho, Iwate, mid May of 1977, and *G. chorda* at Oga-shi, mid September of 1980. Specimens of *S. divaricata* were first procured at Oga-shi, late June of 1976, and weekly from mid June to early August in the next two years and from mid June to mid September in 1979 and 1980.

Toxicity Test

The water soluble and fat soluble fractions were prepared from algal samples by our routine method³⁾ adopted for screening of ciguateric fishes. Each fraction was injected into mice of 18-22 g intraperitoneally at a level equivalent to 2 g of wet seaweed. The fat soluble fraction was given after emulsified with 1% Tween 60 saline solution. The injected mice were placed under observation for 48 h. If two out of three mice in a group were killed, the test solution was regarded to be toxic. A minimum lethal dose was obtained by dilution of an original solution at ratios of 10:5:2:1. Toxicity was expressed by mouse units (MU), where 1 MU is amounts of toxin required to kill a

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Fig. 1. Isolation of toxins A and B from *S. divaricata* collected in 1980 at Oga-shi.

20 g-mouse.

Oral toxicity of synthetic peroxide was examined by administering test solutions emulsified with 1% Tween 60 saline solution through a stomach tube.

Isolation of Toxins from *S. divaricata*

Isolation procedures were shown in Fig. 1. A 1.5 kg portion of minced frozen seaweed was heated with 1.5 l of water at 80°C for 30 min. After cooling 7.5 l of methanol was added to the suspension, which was stirred for 2 h at room temperature and allowed to stand overnight. The mixture was centrifuged, and the residue was twice extracted with methanol. The combined extracts were freed from methanol and extracted five times with an equal volume of diethyl ether. The ether soluble fraction was partitioned between 90% methanol and *n*-hexane. The material in aqueous methanol layer was subjected to dry column chromatography on silica gel (Woelm for dry column chromatography) with 3% methanol in chloroform. The eluates were monitored by thin layer chromatography using Kieselgel 60 precoated plates (Merck, 0.25 mm thick) and chloroform-methanol (95:5). Fractions containing *R_f* 0.45 component were combined and subsequently purified by gel filtration over Bio-Beads S-X12 (Bio-Rad) with chloroform-methanol (2:3) and finally by partition with

a mixture of chloroform, methanol and water (10:5:3).

Fractions containing *R_f* 0.63 component were subjected to vacuum distillation.

Small Scale Isolation of the Principal Toxin from *S. divaricata*

The fat soluble fraction prepared from the specimens collected in 1976 was partitioned with chloroform-methanol-water (10:5:3). The aqueous methanol portion was purified by preparative thin layer chromatography on Kieselgel 60 F₂₅₄ precoated plates (Merck, 2 mm thick) with chloroform-methanol (95:5).

Synthesis of α,α' -Dihydroxy Diethyl Peroxide

The peroxide was synthesized by reacting acetaldehyde with hydrogen peroxide according to the method reported by KHARASCH and SOSNOVSKY⁴⁾.

Spectral Analyses

The infrared spectra (IR) were recorded on a JASCO IR-S spectrometer as films. The ¹H and ¹³C NMR spectra were measured with a JEOL FX-100 spectrometer in deuterochloroform with tetramethylsilane as an internal standard. The EI mass spectra were determined on a Hitachi RMU-6L mass spectrometer and FD mass spectra on a Hitachi RMU-6MG mass spectrometer.

Results and Discussion

Toxicity of *S. divaricata*

Both the fat soluble fractions prepared from *S. divaricata* and *C. okamuranus*, which were collected in 1976, killed mice overnight, whereas the water soluble fractions were nontoxic. The mice injected with toxic extracts showed piloerection, lacrimation, fatigue, paralysis of hind legs, convulsion, respiratory difficulty, and death. It is noteworthy that mice became quite sensitive to physical stimuli. Death time ranged from 30 min to 40 h depending on doses administered.

No toxicity was found in specimens of *S. divaricata* in the next two years, though the seaweed was collected and tested weekly from mid June to early August. It, however, was fortunate that we encountered toxic specimens in late August of 1979 and in mid September of 1980. Toxicity of the former sample was as high as 5 MU/g, while the latter was weakly toxic.

Toxic extracts revealed characteristic stinging odor and white spots on a thin layer plate when exposed to iodine vapor, which were later found to be related to toxicity. Toxicity was detected only when algal samples were heated in the presence of considerable amounts of water. No toxins were extractable either with cold or hot organic solvents. It was also demonstrated that toxicity was steam-distilled directly from seaweeds. It should be noted that lethality was quite labile in an algal body even at -20°C . The first toxic specimen lost activity within a few weeks in a freezer. Surprisingly, the red alga *G. chorda* became nontoxic in a few days, whereas *A. japonicus* maintained

lethality for 3 months. It seems that these differences in stability of toxicity are attributable to their different metabolisms involved.

Isolation and Properties of Toxins

As depicted in Fig. 1, two toxins, A ($R_f 0.63$) and

Table 1. Color tests of natural and synthetic toxins

Reagent	Toxin A	Toxin B	Synthetic toxin
Iodine	—*	—*	—*
Ninhydrin	—*	—*	—*
Dragendorff	—*	—*	—*
Periodate-benzidine	—	+	+
Dimethyl- <i>p</i> -phenylenediamine dihydrochloride	+	+	+
Ammonium thiocyanate-ferrous sulfate	+	+	+
2,7-Diaminofluorene-hemin	+	+	+

* White spot

B ($R_f 0.45$), were isolated pure, though their yields were quite poor. Both toxins were a transparent volatile oil with characteristic pungent odor. Toxin A was lethal to mice at $250\text{ }\mu\text{g/g}$, but toxicity of toxin B was not available due to small amounts. They reacted similarly with various color tests⁵⁾ (Table 1), suggesting the presence of a peroxide moiety. This was also supported by the infrared⁶⁾ (Fig. 2) and NMR spectra^{7,8)}. Toxin A exhibited in ^1H NMR spectrum two methyl signals at δ 1.25 (t, $J=7\text{ Hz}$) and at 1.37 (d, $J=5.4\text{ Hz}$), two nonequivalent methylene protons at 3.69 (dq, $J=16$ and 7 Hz) and at 3.85 (dq, $J=16$ and 7 Hz), a methine proton at 5.04 (q, $J=5\text{ Hz}$), and a hydroxyl proton at 8.56 (br. s). The ^{13}C NMR spectrum

Fig. 2. The infrared spectrum of toxin A (film).

Fig. 3. The infrared spectrum of the principal toxin isolated from *S. divaricata* collected in 1976 at Oga-shi (film).

indicated the presence of a hemiacetal methine carbon at δ 104.3 (d), an oxygen-bearing methylene carbon at 64.1 (t), and two methyls at 18.0 and 15.3 (each q). These data seem consistent with a structure of $\text{CH}_3\text{CH}_2\text{-O-O-CH(OH)CH}_3$ for toxin A. It is noted that no parent ion peak was obtained either in EI and FD mass spectra because of high volatility.

Toxin B showed similar structural features, and spectral analyses [^1H NMR: δ 1.43 (6H, d, $J=5.4$ Hz) and 5.29 (2H, q, $J=5.4$ Hz); ^{13}C NMR: δ 18.5 (q) and 104.0 (d)] suggested a partial structure of $\text{CH}_3\text{CH(O)-O-O-CH(O)CH}_3$. Syntheses of these toxins have not been attempted.

In 1976 we isolated a small amount of the principal toxin from the toxic Oga specimen. The toxin was postulated to be α,α' -dihydroxy diethyl peroxide, $\text{CH}_3\text{CH(O)-O-O-CH(O)CH}_3$, from spectral

data [IR: Fig. 3; ^1H NMR: δ 1.43 (6H, d, $J=5$ Hz), 5.45 (2H, q, $J=5$ Hz), and 8.72 (2H, br. s)]. We then synthesised this peroxide, which resembled the natural toxin in chromatographic and spectral properties [IR: 3300, 1380, 1310, 1155, 1105, 915, 880, 842, and 810 cm^{-1} ; ^1H NMR: δ 1.39 (6H, d, $J=5$ Hz), 5.35 (2H, q, $J=5$ Hz), and 9.73 (2H, br. s); ^{13}C NMR: δ 14.7 (q) and 108.2 (d)]. Color tests were also similar to those for natural toxins (Table 1). The synthetic peroxide showed toxicity in mice of 50 $\mu\text{g/g}$ (i. p.) and of 25 $\mu\text{g/g}$ (oral), and induced in mice signs similar to those by natural toxins. Moreover, these signs seem to be comparable to symptoms of victims poisoned by *mozuku*¹⁾. However, some differences in chemical

Fig. 4. Thin layer chromatograms of toxins A (1) and B (2), synthetic toxin (3), and toxic extracts of *A. japonicus* (4) and *G. chorda* (5).

Samples were chromatographed on a Kieselgel 60 precoated plate (Merck, 0.25 mm thick) with chloroform-methanol (95:5) and the plate was visualized by spraying with dimethyl-*p*-phenylenediamine dihydrochloride reagent.

shifts of NMR and infrared spectra were observed between the synthetic and natural toxins. This may be due to the different stereochemistry at the oxygen-bearing methine.

In addition to *S. divaricata* and *C. okamuranus*, *A. japonicus* and the red alga *G. chorda* were found to possess similar toxins (Fig. 4). This may indicate that a wide variety of marine algae produce similar toxins at a particular stage of life cycle; most likely during reproduction. It is interesting to know whether these toxins play important roles in

their life cycles or not.

We now know that the toxins are produced only when heated in the presence of water. It may be reasonable that the peroxides are responsible for *mozuku* poisoning since these seaweeds are usually eaten after being infused with hot water. Finally, we should emphasize that the toxins are generated under a specific condition. The following equation can be presented;

where X is a precursor. An enzyme would be involved in this reaction. This may explain the rare occurrence of *mozuku* poisoning.

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