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Guam Seaweed Poisoning: Biochemistry of the *Gracilaria* Toxins

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Abstract—Previously undescribed toxic compounds were found to be present at relatively high levels in samples of *Gracilaria tsudai* seaweed collected on Guam soon after a food poisoning outbreak associated with eating that alga. These toxins, for which the names polycavernoside A and B are proposed, were subsequently present in much lower levels in Guam *G. tsudai* samples and have not been identified in seaweed samples from other areas.

It is my pleasure and honor to be here to speak to you about the results of our tests on samples of *Gracilaria tsudai* seaweed from Guam (also known as *G. edulis* and *Polycavernosa tsudai*).

When I received the news about the Guam poisoning incident it did not surprise me because we have had outbreaks in Japan due to ingestion of two different species of *Gracilaria*. In 1980 in Sakata, 2 people became intoxicated after eating *G. chorda* and 1 died (Fusetani & Hashimoto 1984). In 1982, 2 people living in Toyo City became ill after eating *G. verrucosa* and 1 died (Sonoda 1983). In the Akita region 15 people became ill after eating a brown alga, *Nemacystus decipiens*. At Amami island there have been poisoning incidents associated with eating another brown alga.

In Japan *Chondria armata* apparently produces palytoxin for a very short period from the end of June to early July; before and after this period, however, it is very hard to detect this toxin. Another seaweed known to produce bio-active compounds is *Digenea*. This alga produces kainic acid which is known to be anti-helminthic. Neither of these algae are known to have been involved in human food poisoning incidents.

Fortunately, most of our edible seaweed are safe most of the time. Some reports suggest that some species are most likely to be toxic at the end of the reproductive season which, in Japan, is from May to June. After releasing eggs and sperm, they die, detach from the bottom and float away. It is at this time that they may be most toxic. This is apparently what took place in the 2 incidents involving the Japanese *Gracilaria*.

The Japanese have traditionally prepared *Gracilaria* algae by boiling with ash. Nowadays instead of ash we use sodium carbonate which colors the algae a beautiful green. The alkaline condition resulting from this treatment apparently destroys any toxin present. As long as people use these preparation methods and

avoid harvesting seaweed during the reproductive season, eating them is apparently safe. This may be why we seldom see toxicity incidents in Japan in spite of the large amount of seaweed that is consumed.

I would like to emphasize that in these northern areas of Japan it is much too cold for *Palythoa* coral to grow so these poisoning incidents cannot be attributed to contamination from that source.

The symptoms reported in Japanese *Gracilaria* poisoning cases are similar to those which have been described for the Guam cases including nausea, vomiting, diarrhea, stomach cramps, muscle spasm, hypotension and shock (Table 1). Cyanosis is not a significant symptom. Unfortunately the physicians who have treated Japanese patients have not reported these cases in detail in our scientific literature so their symptoms have been gleaned from other accounts and may not be medically correct.

The symptoms reported for brown algae poisoning are slightly different from those described for poisoning due to the *Gracilaria* red algae group; these include malaise, prickling sensation and numbness in fingers and toes and difficulty in respiration. To date no human fatalities have been attributed to ingestion of brown algae.

Table 1. Seaweeds, toxins, symptoms and outbreak data associated with poisoning incidents reported in Japan.

1. *Chondria armata*
   Domoic acid, palytoxin.
   Poisoning incidents not known.
   Amami Islands, Kagoshima Prefecture.
2. *Cladosiphon okamuranus*
   Toxins not identified.
   Malaise, prickling sensation, numbness of extremities, respiratory distress, rash, prosopospasm.
   Yoron, Kagoshima Prefecture, 1974: 5 cases, 0 deaths.
3. *Digenea simplex*
   Kainic acid.
   Poisoning incidents not known.
   Grows in southern part of Japan.
4. *Gracilaria chorda*
   Toxins not identified.
   Nausea, vomiting, diarrhea, hypotension, shock.
   Sakata, Yamagata Prefecture, 1980: 4 cases, 1 death.
5. *Gracilaria verrucosa*
   Toxins not identified.
   Nausea, vomiting, diarrhea, stomach cramps, spasm, hypotension, cyanosis.
   Toyo City, Ehime Prefecture, 1982: 2 cases, 1 death.
6. *Nemacystus decipiens*
   Toxins not identified.
   Vomiting, malaise, stomach cramp, numbness and pain of extremities, cough, eruction.
   Oga City, Akita Prefecture, 1967: 15 cases, 0 deaths.

Although a project has been organized to identify the toxic agent involved in Japanese seaweed poisoning outbreaks, so far it has not been successful. One reason may be that most investigators have extracted algae samples with methanol and did not partition between organic solvent and water soluble components and test each fraction separately. The aqueous fraction has not been shown to be highly toxic to mice in our laboratory. We have found that this agent contains large amounts of potassium and magnesium salts. Potassium salts are highly toxic when injected into mice.

Perhaps one reason we have had difficulty identifying toxins is that they may rapidly disappear from the algae. If the toxin exists in the alga for only a short period, by the time we receive samples and start testing the toxin may have already disappeared. I tested the Guam samples promptly with this in mind. The first sample weighed about 2.75 kilograms and I detected five toxins. One was less polar, dissolved even in hexane, and instantly paralysed mice upon injection. While its effect was immediate, after maybe twenty or thirty minutes most of the affected mice recovered.

The second type of toxin, the components of which we have named Polycavernoside A and B, is the toxin which we believe is responsible for the human fatalities in Guam. These toxins were present in substantial quantities in the first and second samples we obtained from Guam. After June, however, their concentration quickly decreased and we now detect only very small amounts. In contrast, the toxin which we have named XAD2 methanol toxin and which we can purify by using XAD2 resin (it is highly hemolytic and also neurotoxic), is consistently present at low levels. In contrast with polycavernoside A and B, which are apparently present only in the Guam samples, XAD2 methanol toxin is also present in samples collected in Kagoshima and other areas. We suspect these toxins are structurally related but they may not be the main cause for the deaths. We also see differences in mouse symptoms between these two toxin groups.

Extraction of algal toxins is most frequently performed using acetone or methanol. After evaporation of the methanol or acetone the extract is partitioned between dichloromethane and methanol/water. The dichloromethane soluble fraction contains the major toxins, polycavernoside A and B. The XAD2 methanol fraction is obtained by passing the aqueous solution through a XAD2 resin column and then eluting with methanol. This is why we have named this the XAD2 methanol fraction. The amount of polycavenoside sample we have obtained so far is quite small: 200 micrograms of B and 400 micrograms of A. With this small amount we have attempted structure determination and even with modern techniques this has not been easy. However, we have determined that the molecular formula for polycavernoside A is $C_{43}H_{68}O_{15}$ and that the molecular weight is 824.[1]

[1] Following presentation of this talk, we have succeeded in determining the planar structure of polycavernoside A as shown in Fig. 1. (Yotsu-Yamashita et al. 1993)

When these toxins are administered to mice by injection, they may appear normal for fifteen minutes but then a very severe diarrhea starts. We also see hypersalivation and scratching at the face as though the mice are feeling some tingling around the mouth. After they start scratching, paralysis becomes progressively prominent followed by convulsion and spasm. Depending on the dose, the spasms may last for hours, especially in the forelimbs. This symptom is very characteristic of polycavernoside toxicity in the mouse. In contrast, the XAD2 methanol toxin is hemolytic and neurotoxic. It produces paralysis and convulsions but not very much hypersalivation, diarrhea or spasms. These toxins may kill mice in fifteen minutes when a large amount is injected but death generally occurs in twenty-four hours at lower doses.

From spectral data we have determined that polycavernoside A has two sugars which are methylated; most of the sugars have an OH group and most of the time OH groups are in the form of free hydroxyl groups but in this compound the hydroxyl groups are highly methylated. Since the xylose and fucose sugars are quite common among algal products, it is very likely that algae themselves produce this compound. It is not likely that it is produced by *Palythoa* coral or marine bacteria. Another hypothesis: since this compound has both hydrophobic and hydrophylic moieties and since most of the compounds which have this combination of moiety show hemolytic activity, this compound too may be hemolytic. Unfortunately, because the quantity of our purified sample is so limited at the moment, we don't want to use any for testing only hemolysis.

The XAD2 methanol fraction which is always present in the alga is still suspect also. This molecule may have the same structure skeleton as the polycavernoside toxins and differ only in the absence of the methyl or hydroxyl groups. Perhaps when the XAD2 group is methylated it is converted to a more highly

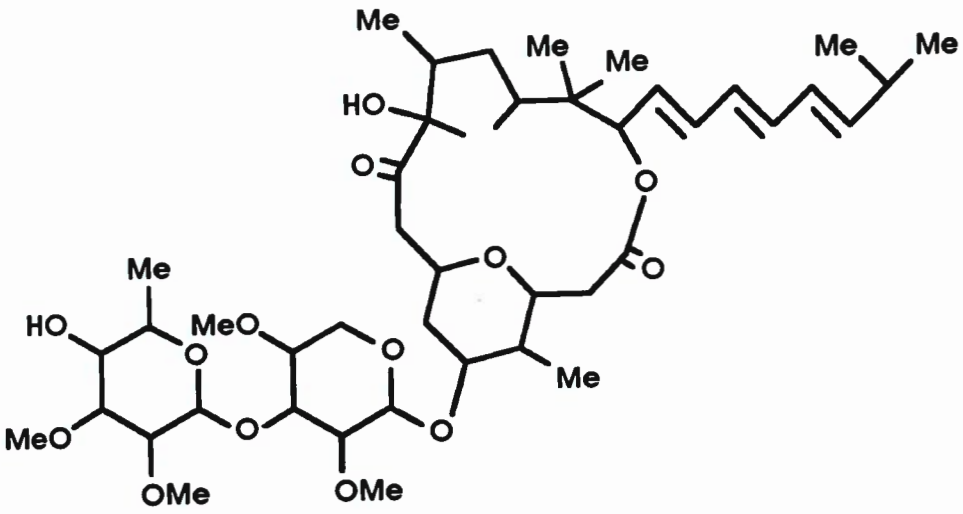

Figure 1. The planar structure of Polycavernoside A.

toxic molecule. At the moment we don't know what physiological or environmental factors may convert a compound of low toxicity to high toxicity.

The polarity of these compounds is also important. If we have a compound with free hydroxyl groups, the structure is similar to the structure of plant saponins and these compounds have highly potent hemolytic properties. Plant saponins alone, however, are not absorbed in the digestive system so when we test this type of compound in the test tube it appears to be more toxic than when it is given orally. Perhaps this methylated compound which has less polarity can be absorbed through digestive membranes and still exert its toxic properties. This is still speculation at this point, however. Perhaps after determining the structure of the toxins we can use this tie-in structure for measuring concentration by HPLC since it has one free hydroxyl group. Perhaps taking advantage of this structure it may be possible to connect these compounds to proteins thereby enabling us to produce toxin-specific antibodies. We are now trying to determine the absolute stereochemistry of these compounds and once that is known a chemist may be able to synthesize them in sufficient quantities to be used in many medical, pharmacological or chemical research projects. A Japanese team has already started synthesis of an important part of this compound and we hope to complete this synthesis when we have determined the stereochemistry of the structure.

The latest sample of Guam *Gracilaria* we obtained in April of 1992 contained only small amounts of these toxins. We saw, however, some slight rise in the concentration of the XAD2 methanol fraction. This suggest there may be some seasonality. We are hoping to obtain more samples in the future so that we may further compare the structure of these toxins. If they have some similarity perhaps they may be precursors of the active toxin. By watching the level of toxin precursors we may then be able to predict when it will be hazardous to harvest the seaweed and we may also be able to predict what physiological, mutational or environmental factors are involved in sudden increases of toxin levels.

While there are still many questions to pursue in the future, with our initial toxin structure determination we at least hope we have cleared the first hurdle in solving this problem. Once synthetic chemists are able to manufacture these compounds, then analytical chemists will be able to determine how to detect them by HPLC and perhaps they can also be tested for by relatively simple tests such as hemolysis inhibition.

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