



TRANSMISSION OF THE PARALYTIC SHELLFISH POISONING TOXINS, FROM DINOFLAGELLATE TO GASTROPOD

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C.-Y. Chen and H.-N. Chou. Transmission of the paralytic shellfish poisoning toxins, from dinoflagellate to gastropod. *Toxicon* **36**, 515–522, 1998.—Purple clams, *Hiatula diphos* Linnaeus, are filter-feeding bivalves and maculated ivory shells, *Babylonia areolata* Link are carnivorous gastropods. Both shellfishes are popular seafood delicacies among the Taiwanese. *Hiatula diphos* were forced to contain gonyautoxins (GTXs) in this research by feeding them with cells of *Alexandrium minutum* Halim, a toxic dinoflagellate species responsible for the paralytic shellfish poisonings in Taiwan. The intoxicated purple clams of known toxicity and toxin composition were fed to *B. areolata* to observe the transmission and transformation of GTXs among this shellfish. It was found that the toxin composition in bivalve and gastropod were similar to that in dinoflagellate. Our data provide evidence for food-chain transmission of paralytic shellfish poisoning toxins, from dinoflagellate to gastropod through a filter-feeding bivalve. The transmitted GTX-I, -II, -III and -IV of *A. minutum* could only be found in the viscera of these shellfish. There was a notable degradation of GTX-I in the ivory shell that resulted in a decrease in toxicity while the total amount of toxins was accumulatively increasing. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Among all seafood poisonings, paralytic shellfish poisoning (PSP) poses the most serious threat to public health due to the extreme toxicities of the toxins involved. Economic damages caused by the toxins accumulated in shellfish through filter-feeding on toxic algae can be immeasurable (Shumway, 1990). Toxins responsible for PSP are produced by a number of dinoflagellate species, especially *Alexandrium tamarense*. A toxic strain of *A. tamarense* was reported to contain gonyautoxins (GTXs) that caused several seafood poisoning incidents in Taiwan, by passage through the purple clam, *Hiatula diphos* Linnaeus (Su *et al.*, 1989). This toxic dinoflagellate was later reidentified

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as *A. minutum* Halim according to Dr D. Anderson, Woods Hole Institute of Oceanography, U.S.A. (personal communication). Several strains of this dinoflagellate species including toxic and nontoxic ones have been isolated from local waters and maintained as unialgal cultures in our laboratory.

Bivalves are filter-feeding shellfish that are thought to be the most important vectors of PSP. However, there are several reports indicating that many carnivorous and scavenging gastropods are also carriers of PSP toxins (White *et al.*, 1993; Hwang *et al.*, 1994). The origin of these PSP toxins in gastropods is unclear yet due to the lack of evidence of a direct connection between toxic phytoplankton and carnivorous shellfish. Although it has been suggested that gastropods acquire their toxins through feeding on toxin-contaminated filter-feeding bivalves (Shumway, 1995), more observations or experiments on the mechanism of food-chain accumulation are needed.

In this study we fed purple clams (*H. diphos*) with cultures of the toxic dinoflagellate *A. minutum*, which has been reported to have a toxicity of 8.3×10^{-5} MU per cell (Chou and Su, 1989), to an extent that the accumulated toxins could be accurately measured. Various amounts of the toxic bivalves were then fed to different groups of carnivorous gastropods, *Babylonia areolata* Link. The transmission of toxins from bivalve to gastropod and changes in toxin composition were shown by toxin profile analysis of groups of ivory shells kept for different holding periods after feeding upon the dinoflagellate fed bivalves.

MATERIALS AND METHODS

The strain AMTK-1 of *A. minutum* was isolated in 1986 from the Tungkang area where PSP occurred due to the ingestion of purple clams. Unialgal cultures were kept in K-medium and illuminated with continuous light of 3000 lux at 20–22°C. *Isochrysis* sp. cultures, used as nontoxic live feed for control purposes, were also maintained under the same culture conditions. Algal cells of both toxic and nontoxic species were harvested for feeding experiments at their exponential growth phase.

Purple clams (*Hiatula diphos*), size range 6–8 cm, were collected from an aquaculture pond in Chigu, Tainan Prefecture. Fifty animals were brought to the laboratory and divided into two 130 l tanks of filtered sea water. Aeration at ambient temperature and water salinity were maintained constant throughout the holding period. Half of the purple clams were fed with the toxic dinoflagellate (total: 1.66×10^9 cells) on the first and third day, while the other half (control group) were fed with nontoxic algae (total: 1.61×10^9 cells) on the same dates. Algal cells taken by each of the purple clams were calculated from daily counts of cell concentration in the different tanks.

A carnivorous gastropod, *Babylonia areolata* (shell length 6.5–8.5 cm), was bought from Keelung Fish Market and tested to be free of PSP toxins by random samplings and a high performance liquid chromatography (HPLC) analysis. Twelve animals were placed in two 130 l sea water tanks equipped with aeration and circulation systems. They were starved for 1 week before the feeding experiment. The culture conditions for gastropods were kept the same as for purple clams, except that the animals were isolated from each other by netted frames. Each gastropod was fed with one purple clam every 2 days three times except for animals that were killed for toxin analysis, 1, 2 and 3 days after each feeding. The experimental group of six gastropods was fed with toxic purple clams of known toxicity and toxin composition which had fed upon toxic dinoflagellates, whilst the controls were fed with nontoxic purple clams which had fed upon *Isochrysis*.

Toxin compositions and toxicities of purple clams were analysed on the same day they were fed to the gastropods. Two animals were randomly sampled for this analysis from either the toxic or the nontoxic groups of purple clams on the same day of feeding to the ivory shells to represent the status of the whole population. Similar analyses were performed on the ivory shells sampled on the second day, the third day and the fourth day of respective serial feedings of toxic and nontoxic purple clams, two animals as replication for each analysis. Extraction of the toxic components in shellfish was as previously described by Su and Chou (1993). Whole bodies of purple clams and separated portions (digestive gland and muscle) of ivory shell were homogenized and extracted in a solution of 0.1 N HCl. Toxin extracts were further defatted with chloroform twice and the aqueous layer were filtered through an ultrafiltration membrane (10 000 MW cut-off) by centrifugal force. Ten microliters of filtrate from each sample was subjected to HPLC analysis that was modified from Nagashima *et al.* (1987). The reverse phase liquid chromatography used a Cosmosil 5C18-AR column (Nacalai Tesque, Japan), 250 × 4.6 mm, with 0.05 M phosphate buffer (pH = 7.0) which contained 2 mM HSA (heptanesulfonic acid, H8901; Sigma, USA) as mobile phase, with a flow rate of 0.8 ml min⁻¹.

Fluorescent GTX derivatives from a post-column reaction were detected using a Hitachi F-1000 spectrophotometer (Hitachi Ltd., Japan) with excitation setting at 336 nm and emission at 390 nm.

The conversion into mouse units of peak areas of HPLC-resolved toxins was according to the following formulation: toxicity (MU ml^{-1}) = $(19.7A_1 + 0.57A_2 + 1.61A_3 + 8.11A_4) \times 10^{-6}$, where A_1 , A_2 , A_3 and A_4 represented the peak areas of the resolved toxins, GTX-I, II, III and IV, respectively. The feasibility of the formula was based on the fact that there were no toxins other than the above four in the experimental material. In addition, the correlation factors for each toxin were calibrated from the mouse units assay of the partially purified toxin mixture with the reported specific toxicity (Genenah and Shimizu, 1981) and specific fluorescent response (Sullivan *et al.*, 1985). In spite of a lack of available authentic GTXs, this provides a way to convert the HPLC data to the generally accepted mouse toxicity, expressed as STX (saxitoxin) equivalents (Williams, 1984). In our case it was observed that 1 MU (ICR strain, male, 20 g) was equivalent to $0.259 \mu\text{g ml}^{-1}$ STX by the calibration procedure suggested by the A.O.A.C. (Su and Chou, 1993). According to the above formula and the corresponding mouse responses to each toxin, the specific content (μmol) of each individual toxin could thus be calculated from its peak area. Correlation factors for each toxin were 1.2×10^{-10} , 7.2×10^{-12} , 7.2×10^{-12} and $1.2 \times 10^{-10} \mu\text{mol}$ per area unit for GTX-I, -II, -III and -IV, respectively.

RESULTS

Average toxicities accumulated in each clam were presented by the total mouse units that were converted from HPLC analysis of the shellfish extracts and the accumulated cells of toxic dinoflagellates taken by their filter-feeding activities (Fig. 1). Toxicities of purple clams were analysed after 5, 7 and 9 days exposure to the toxic algae at a concentration of *ca* $5 \times 10^5 \text{ cells l}^{-1}$. It was found that purple clams gained their toxicities quickly (2700 MU per shellfish on the sixth day and 4333 MU per shellfish at the tenth day) and became inedible. According to Williams (1984) and current regulation (Hallegraeff *et al.*, 1995), the PSP trace allowable in seafood is $80 \mu\text{g}$ STX equivalent/

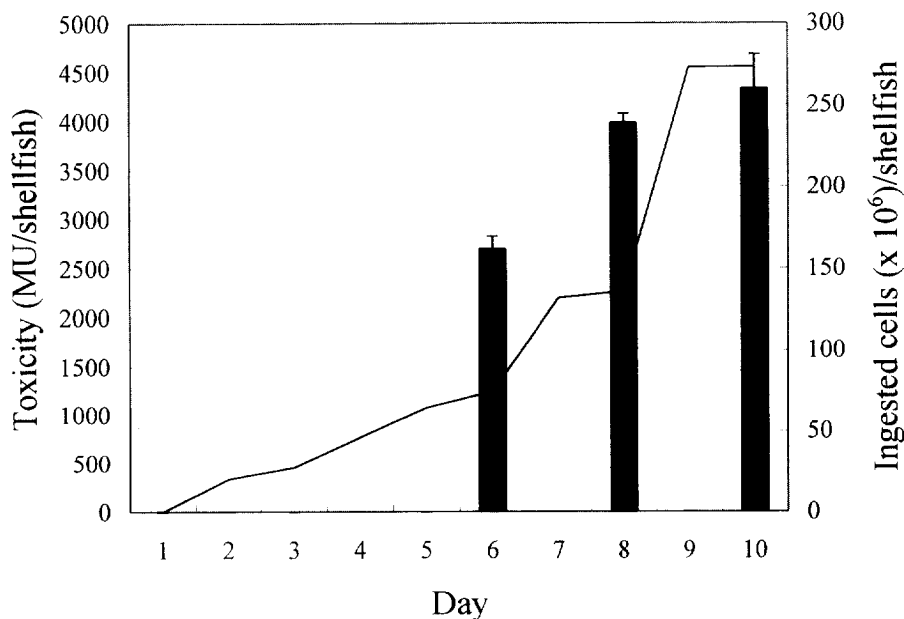


Fig. 1. The amount of ingested toxic dinoflagellate (*A. minutum*) cells (line) and the toxicity of mouse unit detected in purple clams (*H. diplos*) during the filter-feeding period (bar). Algal cells taken by each of the purple clams were calculated from the daily reduction of the cells. Values of toxicity are means \pm SE converted from HPLC analysis of two replications.

100 g tissue, compared with *ca* 309 MU/100 g tissue in this case. It was also found that the toxicities converted from the HPLC data of existing toxins in shellfish were lower than those calculated from the actual intake of toxic *A. minutum* cells.

Algal cells taken by the purple clam were calculated from the daily reduction of the cell concentration for the entire 10 days of the feeding experiment. Then the total toxicity taken through the toxic dinoflagellate was calculated from the accumulated cells per shellfish and an averaged toxicity of 8.3×10^{-5} MU per cell derived from HPLC and mouse assays (Chou and Su, 1989). The toxin compositions of purple clam groups were

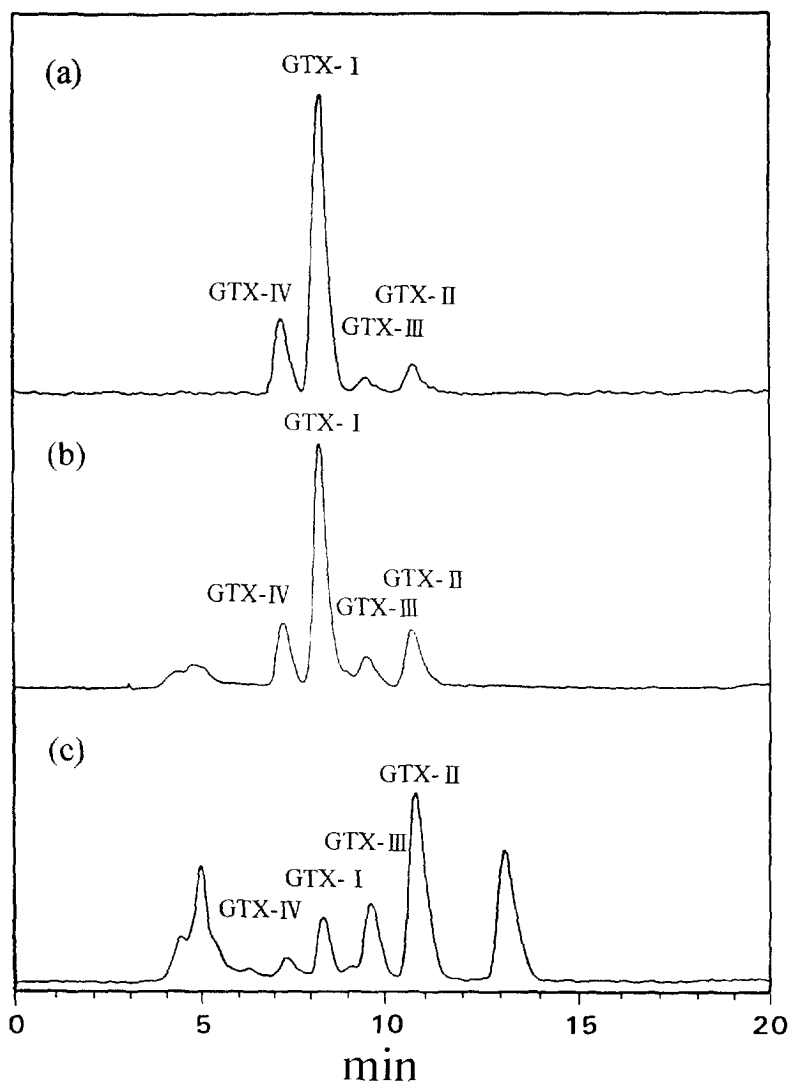


Fig. 2. Composition of GTX-I, -II, -III and -IV identified in (a) dinoflagellate (*A. minutum*), (b) purple clams (*H. diplos*) and (c) ivory shells (*B. arcolata*) by HPLC using a Cosmosil 5C18-AR column, 250 × 4.6 mm, and a mobile phase of 0.05 M phosphate buffer (pH = 7.0) containing 2 mM HSA, flow rate of 0.8 ml min⁻¹.

also obtainable from HPLC analysis. Figure 2 shows HPLC chromatograms of toxins in dinoflagellates, purple clams and ivory shells that feed on toxic algae or toxin-contaminated purple clams. GTX-I, -II, -III and -IV were found in similar relative amounts in the experimental algae and shellfishes. All the sample extracts contained trace and almost equal amount of GTX-III and -II in addition to the two major toxins GTX-I and -IV. There were no detectable quantities of other GTX or saxitoxin derivatives in our samples. Purple clams after different incubation periods with *A. minutum* were analysed for their specific toxin contents. Figure 3 showed the contents of GTX-I and -IV increased with the incubation time, but not GTX-III and -II. It seems that GTX-II and -III have a shorter half-life than GTX-I and -IV when accumulated in purple clams.

Ivory shells that were fed on more toxin-containing purple clams showed higher toxin contents, especially the content of GTX-IV (Fig. 4). However, due to a significant transformation of GTX-I in ivory shells, the overall toxicity of the ivory shells of the third group showed a lower level than that of the second group (Fig. 4). GTX-I content did not follow the same increasing amount as other toxin analogues in the different experimental groups, although the second group had taken more toxic clams than the first group and the third group even more. The result indicated that the PSP toxins of the dinoflagellates were transferred to the purple clams by their filter-feeding activities and then transmitted to the carnivorous ivory shells through the preying activities on the toxic bivalves. Although the ingested toxins could be transformed or degraded to be less in quantity or less toxic in both shellfishes, they could also accumulate and remain in the animals for some time. It was also noted that most of the toxins taken from food by the ivory shells remained in their digestive glands without being transferred to other muscle tissues.

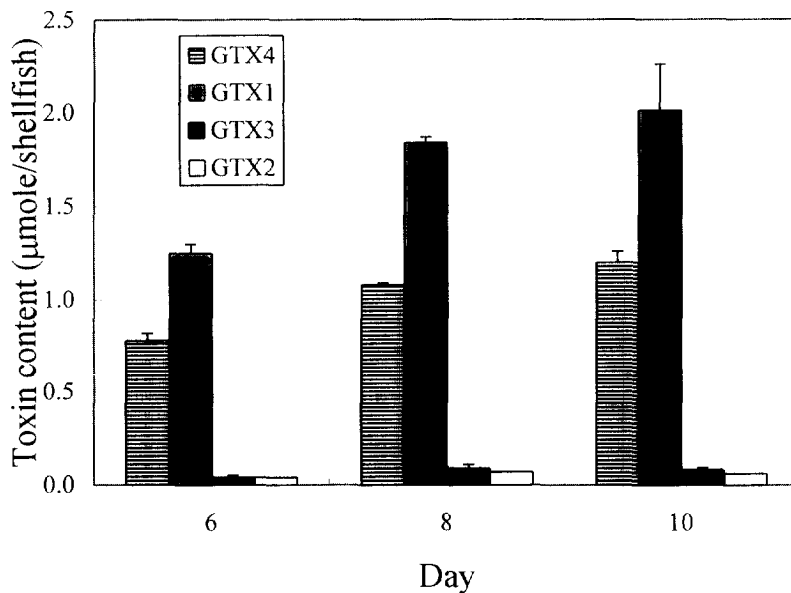


Fig. 3. Changes of PSP toxin compositions in purple clams (*H. diplos*) during the feeding period of dinoflagellate (*A. minutum*) cells. Values are means \pm SE of two replications.

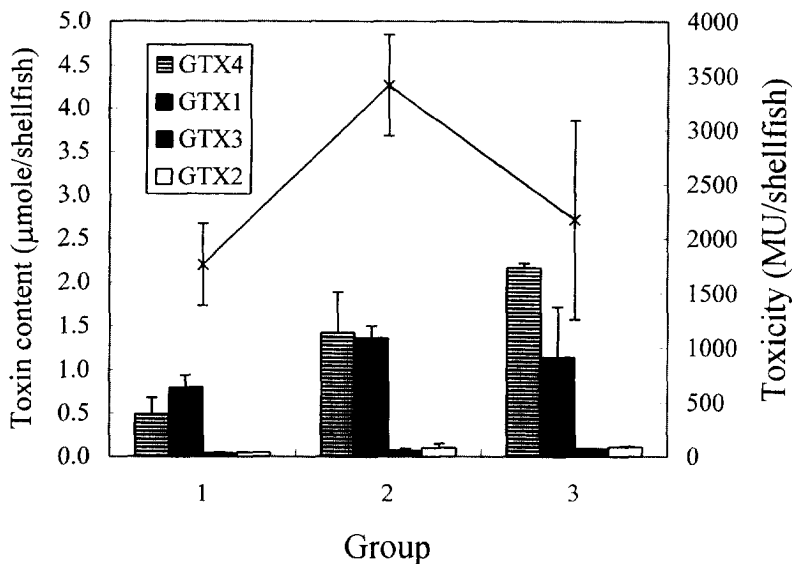


Fig. 4. Toxin compositions (bar) and toxicities (line) in three groups of ivory shells (*B. areolata*) that fed on the increasing amount of toxin-contaminated purple clams. Values of both toxin content and toxicity of each shellfish group are means \pm SE of two replications.

DISCUSSION

In Taiwan, purple clam (*H. diphos*) is the only species known to be involved in PSP, due to its higher efficiency in accumulating and retaining *A. minutum* toxins than other culture bivalves such as *Crassostrea gigas* and *Meretrix lusoria* (Chou and Su, 1989). From our records it seems that only the cultured animals may cause the endemic problems, while the wild purple clams seldom contain detectable quantities of toxins. The ivory shell (*B. areolata*) is one of the economically most important shellfishes in Taiwan. They have never been reported as toxic except one of their related species is containing tetrodotoxin (Lin *et al.*, 1996). Hwang *et al.* (1994) also reported that the gastropod *Niotha clathrata* collected from the area where the purple clams were cultured contained trace amounts of GTX-III in addition to tetrodotoxins. The authors of this paper postulated that the origin of GTX in *N. clathrata* might be from purple clams or other bivalves. There are also many other reports demonstrating the carnivorous gastropods obtain their PSP toxins from consuming filter-feeders that grow in an environment rich in toxic dinoflagellates (Shumway, 1995). However, there has been a lack of experiments to show transmission of these toxins from filter-feeding bivalves to gastropods, or toxin transformation inside the gastropods. We used *B. areolata* to feed on the toxin-contaminated purple clams to observe the preying activity and the toxin transmission. The use of PSP-unrelated *B. areolata* instead of *N. clathrata* in this experiment was based on the fact that *B. areolata* are larger in size and more aggressive predators. A complete swallowing of purple clam tissue was observed in *B. areolata*.

Our results present direct evidence for the transmission of dinoflagellate PSP toxins to carnivorous gastropod through bivalve mollusc vectors. Both the intoxicated *H. diphos* and *B. areolata* did not show any adverse effects from the GTXs of *A. minutum*.

Secondary intoxication of gastropods poses an extra threat to shellfish consumers. Hence, not only the bivalves, but also the gastropods have to be monitored for toxic risk when *A. minutum* blooms in a harvesting area. Present results also show that *B. areolata* concentrated the toxins acquired from the purple clam only in the digestive gland, without contaminating other muscular portions. Caddy and Chandler (1968) also found that the digestive gland was usually toxic and the muscular tissue was toxin-free in the wild specimens of *Buccinum undatum*.

Toxin compositions in purple clams and ivory shells were found to be the same as in dinoflagellates except for the last sample group of ivory shell. It seems that the purple clam and ivory shell digest or transform the toxins in different manners. We have seen a rather rapid depuration of GTXs in purple clams after the termination of feeding with toxic dinoflagellates (unpublished data). In the present experiment we did not wait long enough to see a complete depuration of GTXs in the ivory shell. However, we have observed the transformation of GTX-I to a lesser amount and lesser toxicity in animals left 3 days for depuration. Transformation of PSP toxins in shellfish has been studied and reported in many papers (Sullivan *et al.*, 1983; Lassus *et al.*, 1989; Lassus and Bardouil, 1996). The degradation and transformation may be undertaken by enzymes of the shellfish itself or by microbes in the gut of the predators (Kotaki *et al.*, 1985). Toxin compositions in different algal strains and bivalves were reported as differing from each other (Lassus *et al.*, 1989; Cembella *et al.*, 1994). Hwang *et al.* (1987) reported a trace amount of STX and neoSTX in the purple clams of a field collection while an *A. tamar-ense* (supposed to be *A. minutum*) bloom occurred. However, from this experiment and other results of our laboratory works we have seldom detected the saxitoxins and other GTX analogues beside the reported GTX-I ~ -IV in the cultured strains of *A. minutum* or in the purple clams collected.

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