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Difference in PSP Composition among Various Parts of Surf Clam
(Received October 30, 1998)

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Surf clam Pseudocardium sachalinensis was collected from coastal waters of Ibaraki Prefecture and dissected into various parts, which were analyzed for paralytic shellfish poisons (PSPs) by HPLC. In all the parts, PSP was composed almost exclusively of protogonyautoxin (PX) and gonyautoxins (GTXs) 1–4. The relative amounts of those components, however, differed among the parts. Overall, the ratio of GTX(1,4) (N1-OH carbamate toxins) to GTX(2,3) (N1-H carbamate toxins) clearly differed between the visceral parts and the muscle parts, suggesting the involvement of enzymatic reaction. When added to the visceral extract, GTX (1,4) (N1-OH toxins) was for the most part converted into GTX(2,3) (N1-H toxins). This, along with some other data, suggested that biotransformation of GTX(1,4) to GTX(2,3) takes place in surf clam viscera.

Key words: paralytic shellfish poison (PSP); surf clam; HPLC; PSP composition; enzymatic reaction

Paralytic shellfish poison (PSP) is a potent biotoxin which is produced by some dinoflagellate planktons. This toxin is composed of more than 20 components which differ from one another both in structure and toxicity (Fig. 1). Bivalves are plankton feeders and this can result in excessive PSP accumulation. Such toxicity poses a serious problem to public health, and also to the fishery industry, especially shellfish-farming.

In a previous paper¹, we described the PSP infestation to two commercially important bivalves (hard and surf clams), as well as mussel, in Ibaraki Prefecture. We also found clear differences in PSP composition among the three bivalves, and even between visceral and muscular parts excised from each clam. These findings are helpful, not only for elucidating PSP metabolism, but also from a food-hygienic point of view, since such conversion of PSP components may change the total toxicity of the bivalve.

In this situation, the present study was undertaken to examine the PSP composition in various anatomical parts of surf clam. Attempts were also made to confirm the involvement of enzyme(s) in the conversion of PSP.

Materials and Methods

Materials
Specimens of the surf clam Pseudocardium sachalinensis were collected at Kujihama, Ibaraki Prefecture in April, 1996. Thirty specimens were dissected into digestive gland, intestine, gonad, crystalline style, other viscera, gill, mantle, limb of mantle, siphon, adductor muscle, foot and other muscles. Each part was pooled and kept at −40°C until assay. Some specimens were divided into the visceral and muscular parts and the visceral part was used in enzymatic experiments.

Reagents
All solvents and reagents were of analytical grade.

PSP standard—Gonyautoxin (GTX) 1–4 mixture was prepared from PSP-infested scallop Patinopecten yessoensis by the method of Noguchi et al², and used as the standard for PSP composition analysis. A GTX1–4 mixture was purchased from Wako Pure Chemical Industries
and used for enzymatic experiments as the 'substrate'. A GTX 1, 4 mixture was provided by Dr. O. Arakawa, Kagoshima University.

**Assay of toxicity and determination of PSP composition**

Toxicity was determined by mouse assay\(^3\). PSPs from various parts of surf clam were extracted as described in the previous paper\(^1\) and their composition was examined by post column derivatization high performance liquid chromatography (HPLC)\(^5\). After hydrolysis of the test solution with diluted hydrochloric acid in boiling water for 15 min, HPLC analysis was also performed for protogonyautoxins (PXs). The value for PXs was estimated by subtraction of the value for the corresponding GTXs before hydrolysis from that after it.

**Conversion of GTXs in bivalves**

The visceral part was homogenized with an equal volume of 0.05 mol/L sodium phosphate buffer (pH 6.8). The homogenate was centrifuged at 10,000×g for 10 min. The antibiotic gentamicin (1 mg/mL) was added to part of the supernatant. Otherwise, the supernatant was filtered through a 0.22 μm filter. Then 10 μL of GTX1-4 mixture or GTX1, 4 mixture was added to 500 μL of the above supernatant or filtrate and the solution was held at 12°C. At intervals, small aliquots of the solution were taken and analyzed by HPLC. The effects of (1) pH (2.5 to 7.0), (2) heating for 10 min at 60°C, and (3) dialysis using a membrane (MWCO 14,000), on the conversion of GTXs were also examined. The conversion experiments were performed with sterilized implements at a clean bench.

**Results**

PSP composition profiles of various anatomical parts from surf clam are shown in Fig. 2.

The various visceral parts except the crystalline style contained much less N1-OH toxins (GTX1, 4) than N1-H toxins (GTX2, 3), whereas GTX1, 4 were predominant in the muscular parts such as foot and adductor muscle. The crystalline style showed a ratio of GTX 1, 4 to GTX2, 3 close to that of the muscular parts. The ratios of GTX1, 4 to GTX2, 3 in gill and mantle were intermediate between those in the visceral parts of the surf clam.
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The profiles are rather part- or organ-specific. Overall, the ratio of GTX1, 4 to GTX2, 3 differed markedly between visceral and muscular parts, as noted in the previous paper\(^1\). Table 1 shows the ratios in twelve anatomical parts, along with their toxicity scores. "Other viscera" (excluding digestive gland, gonad and intestine) gave the lowest ratio of 0.89, followed by digestive gland (0.96), intestine (1.36), gonad (1.79), gill (2.59) and mantle (3.21).

When a GTX1, 4 mixture was added to the visceral extract of surf clam, both GTX1 and GTX4 peaks decreased with increase of the reac-
tion time, while the GTX3 and GTX2 peaks became larger. The PXs peak did not change markedly before and after the reaction. Figure 3 shows these time-dependent changes in the HPLC PSP profile.

Next, the visceral extract was diluted with 9 vols of 0.05 mol/L phosphate buffer (pH 6.8) and then 10 μL of GTX1-4 was added to 500 μL of the solution. HPLC analysis demonstrated that GTX1 and GTX4 were almost quantitatively converted to GTX2 and GTX3, respectively (Fig. 4). GTX4 disappeared after 120 min and GTX1 after 180 min. Figure 5 shows the time-dependent changes of the four GTXs. The extracts from the digestive gland, intestine, and gonad did not show such converting activity (data not shown).

Part of the visceral extract was dialyzed using a membrane (MWCO 14,000) against 0.05 mol/L phosphate buffer (pH 6.8). HPLC analysis showed that the dialysis did not affect the conversion of GTXs (Fig. 6). When heated at 60°C for 10 min, the visceral extract almost completely lost the converting activity. These results suggest that enzymes may be involved in the conversion reaction.

Part of the visceral extract was divided into several lots, each of which was adjusted to a given pH between 2.5 and 7. Each solution was mixed with GTX standard, left for 60 min at 12°C, and analyzed for the converting activity. As shown in Fig. 7, the activity was hardly detected at pH 2.5 but increased almost in parallel with the increase of pH.

**Discussion**

It has been reported that specimens of a bivalve species exhibit somewhat different PSP patterns even when collected from the same site at the same time. As described previously, surf clam toxin was composed almost exclusively of GTX1-4 and PXs, as were those of the hard clam and mussel. The PSP composition profiles of both types of clams resembled each other, differ-
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ing clearly from that of mussel with a high GTX (1,4)/GTX (2,3) ratio. A similar difference in the ratio was found between the visceral and muscular parts of surf clam; the visceral part exhibited a prominent GTX3 peak, along with much smaller peaks of GTX 1, 2, 4, whereas the profile of the muscle part exhibited approximately similar peaks of GTX1-4. On the other hand, it is generally accepted that the digestive gland of bivalve can accumulate PSP at a high concentration. In this study, PSPs in different anatomical parts of surf clam were also composed almost exclusively of GTX1-4 and PXs, but differed from one another in toxicity as well as PSP composition profile. The ratio of GTX(1, 4) to GTX(2, 3) differed among the species and further among the anatomical parts of surf clam. GTX1, 4 levels were lower in various visceral parts than in the muscular parts. Addition of GTX1, 4 to the visceral extract did not increase the ratio of GTX1, 4 to GTX2, 3. These results suggested that PSP conversion occurs in surf clam viscera.

We then examined whether the conversion was enzymatic or not. The visceral extract did not show any PSP-converting activity when preheated at 60°C for 10 min. Pretreatment of the extract at low pH (down to 2.5), resulted in a significant loss of the activity. On the other hand, the visceral extract retained PSP-converting activity when dialyzed using a membrane (MWCO 14,000). All these results could support the occurrence of enzymatic conversion from GTX1 and GTX4 to corresponding N1-deoxygenated analogues GTX2 and GTX3, respectively. Other parts of the surf clam, such as gill, mantle and muscular parts, whose GTX(1, 4)/GTX(2, 3) ratios were high, did not show any conversion even after 24 hrs.

Kotaki et al. isolated some bacteria that converted GTX to STX from coral reef and marine snail. In our study, the reaction was performed under bacteriostatic conditions, so, involvement of bacteria in the reaction is likely to be negligible, if any.

A few studies have been carried out on the enzymatic conversion of PSP in bivalves, using

Fig. 4. Transformation of GTX (1, 4) to GTX (2, 3) in surf clam
a) Immediately after adding GTX1–4 to the visceral extract which was previously diluted with 9 volumes of 0.05 mol/L phosphate buffer (pH 6.8). b) After 30 min, c) After 60 min, d) After 120 min, e) After 180 min
4: GTX4, 1:GTX1, 3:GTX3, 2:GTX2

Fig. 5. Time-dependent transformation of GTX (1, 4) to GTX (2, 3) in the visceral extract from surf clam

![Graph showing transformation of GTX (1, 4) to GTX (2, 3) over time.](image)
crude tissue homogenates\textsuperscript{7-9}. Shimizu and Yoshioka\textsuperscript{7} supposed that N1-hydroxyl group reduction in sea scallop Placopecten magellanicus homogenates, particularly of locomotory tissues, is mediated enzymatically. Sullivan et al\textsuperscript{8} reported enzymatic decarbamoylation of PSP in the littleneck clam Protothaca staminea. Oshima\textsuperscript{9} found an enzyme which catalyzed hydrolysis of the N-sulfocarbamoyl moiety and converted PX (C)1, 2 to dcGTX2, 3 in two species of clam, Mactra chinensis and Peronidia venulosa. However, he was unable to isolate any enzyme involved in the conversion of the N1-hydroxy group (GTX 1 and GTX4). Finally, he concluded that the conversion could take place in the presence of natural reductants such as glutathione and cysteine. The conversion by means of such reductants, however, would take a much longer time and require more severe conditions than the enzyme-associated conversion, indirectly supporting the idea that some enzyme(s) could be involved in the PSP conversion.

It is important to proceed further with these lines of study on bivalve PSP, since the total toxicity of bivalves may be changed by such enzymatic conversion. In this connection, isolation and characterization of this enzyme(s) remain to be performed.

\textbf{Acknowledgment}

We thank Mr. M. Okawa and Mr. M. Fujitomi, Ibaraki Prefectural Fisheries Experimental Station for their help in collecting surf clam speci-
mens.

References