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ENZYMATIC TRANSFORMATION OF PSP TOXINS IN THE LITTLENECK CLAM (*Protothaca staminea*)

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SUMMARY: Stemming from investigations into the relationship between toxins produced by *Gonyaulax* sp. and accumulated in shellfish, we wish to report enzymatic transformations of the PSP toxins to decarbamoyl derivatives in the littleneck clam (*Protothaca staminea*). No toxin transformations were observed in either mussels (*Mytilus edulis*) or in butter clams (*Saxidomus giganteus*). In addition, littleneck clam samples from the natural environment contained predominantly the decarbamoyl derivatives, while other shellfish species collected from the same vicinity contained the previously reported PSP toxins.

Paralytic shellfish poisoning (PSP), a problem endemic to many of the temperate coastal areas of the world, involves the accumulation of potent neurotoxins of dinoflagellate origin (*Gonyaulax* sp.) in bivalve molluscs. Originally it was thought that only one toxin, saxitoxin (STX), was involved, but it is now known that several closely related toxins, the gonyautoxins (GTX I-IV) and neosaxitoxin (NEO), are present in toxic shellfish from many parts of the world (1-4) (see figure 1). Recently it has been reported that PSP toxins in several strains of *Gonyaulax* sp. occur predominantly as the N-sulfocarbamoyl derivatives (B1, B2, C1-C4 in figure 1) (5-7), and these toxins have recently been reported in shellfish (8). It is apparent that the development of toxicity in shellfish is extremely complex, involving a large number of related toxins. Several studies addressing toxin distributions have noted differences in the relative levels of the various toxins between dinoflagellates and shellfish (9, 10) and between different species of shellfish collected in the same area (3). Since the toxins are all closely related structurally, metabolic interconversions of the toxins may be the reason for the dissimilarity in toxin profiles. Recently, transfor-

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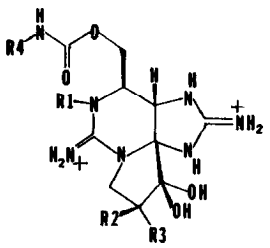
P.S.P. Toxins



R1	R2	R3	Carbamate Toxins (R4-H)	N-Sulfocarbamoyl Toxins (R4-SO_3^-)
H	H	H	STX	B1
OH	H	H	NEO	B2
OH	H	OSO_3^-	GTX I	C3
H	H	OSO_3^-	GTX II	C1
H	OSO_3^-	H	GTX III	C2
OH	OSO_3^-	H	GTX IV	C4

Figure 1. Toxins associated with paralytic shellfish poisoning (GTX-gonyautoxin, NEO-neosaxitoxin, STX-saxitoxin). Toxins C3 and C4 have not been reported but are postulated N-sulfocarbamoyl forms of GTX-I and GTX-IV respectively.

mations involving the conversion of the gonyautoxins and neosaxitoxin to saxitoxin have been reported in one species of scallop (*Placopecten magellanicus*) (11). We wish to report on the occurrence of metabolic conversion of the PSP toxins in littleneck clam (*Protothaca staminea*) tissue homogenates and on the distribution of the toxin metabolites in various bivalve species in Puget Sound, Washington.

MATERIALS AND METHODS

Toxic and nontoxic littleneck clams (*Protothaca staminea*), mussels (*Mytilus edulis*), and butter clams (*Saxidomus giganteus*) were collected from beaches on Puget Sound, Washington. All samples were either processed immediately after collection or held live for a short time in aquaria (12°C) until utilized. Investigations into the metabolic transformations of the PSP toxins were conducted by incubating purified toxins in tissue homogenates (Xg tissue + 6X ml H_2O, pH 6.2) at 30°C for periods of time ranging up to 24 hr. At the end of the incubation period, enzymatic activity was stopped by adding 4 volumes 95% methanol (pH 4.0). The toxin content before and after incubation was determined by high pressure liquid chromatography (HPLC) using both amino and cyano bonded phase columns (12).

Decarbamoyl derivatives of the PSP toxins were prepared chemically by strong acid hydrolysis (7.5 N HCl) (13) and carbamoylation reactions were carried out according to the procedures described by Koehn *et al.* (14). Toxicity testing of the purified toxins and tissue homogenates was performed by the mouse bioassay (15) using 19-22 g Swiss-Webster mice.

The occurrence of the various PSP toxins in the natural environment was investigated by analyzing dinoflagellates and naturally contaminated shellfish from Puget Sound, Washington. Shellfish samples were extracted with 2 volumes .05N HCl, an aliquot of the supernate diluted with an equal volume metanol, and analyzed by HPLC as previously described. A similar procedure was used for dinoflagellate cells (collected in a 28 µm net) except acetic acid was substituted for HCl.

RESULTS AND DISCUSSION

Incubation of the N-sulfocarbamoyl toxins (B1, C1, C2) in littleneck clam homogenates effected transformation of the toxins. The products of the reaction were chromatographically distinct from all of the PSP toxins reported to date, showing a somewhat longer retention time on the amino column than the carbamate toxins (see figure 2). It was found that the new compounds (designated STX-M, GTX-IIM and GTX-IIIM from B1, C1, and C2 respectively) were formed most rapidly in homogenates of the littleneck clam viscera (ca 90% completion in 4 hours) with decreasing rates of formation in the mantle, muscle, and siphon homogenates. In addition, it was found that the same products (STX-M, GTX-IIM, GTX-IIIM) were formed by incubation of the carbamate PSP toxins (STX, GTX-II, GTX-III) (figure 2, A) with littleneck clam homogenates, although the reaction was much slower than in the case of the N-sulfocarbamoyl toxins, suggesting that the sulfo group on the carbamate is somehow involved in the enzymatic reaction. Evidence that this transformation is enzymatic rests with the observations that heat inactivation of the homogenates (boiling), adding organic solvents (methanol), or low pH effectively block the reaction. In the case of the butter clam and mussel homogenates, no transformation occurred even after 24 hr and the N-sulfocarbamoyl and carbamate toxins were recovered intact at the end of the incubation period.

The enzymatic transformations described above result in formation of compounds that have toxicities intermediate between the N-sulfocarbamoyl and carbamate toxins. The reported toxicities of C1, B1, GTX-II and STX are ca 15, 150, 1050 and 2300 MU/μmole (6,7). (One mouse unit or MU is the amount of toxin needed to kill a 20g mouse in 15 min.) The toxicities of the C1 metabolite (GTX-IIM) and the B1 metabolite (STX-M) are approximately 380 ± 50 MU/μmole and 900 ± 120 MU/μmole respectively. (The toxicities of GTX-IIM and STX-M were determined by correlating the reaction mixture toxicity with the toxin content as measured by HPLC, and assuming that the fluorescence yields for the metabolites are the same as for the carbamate toxins.) This has

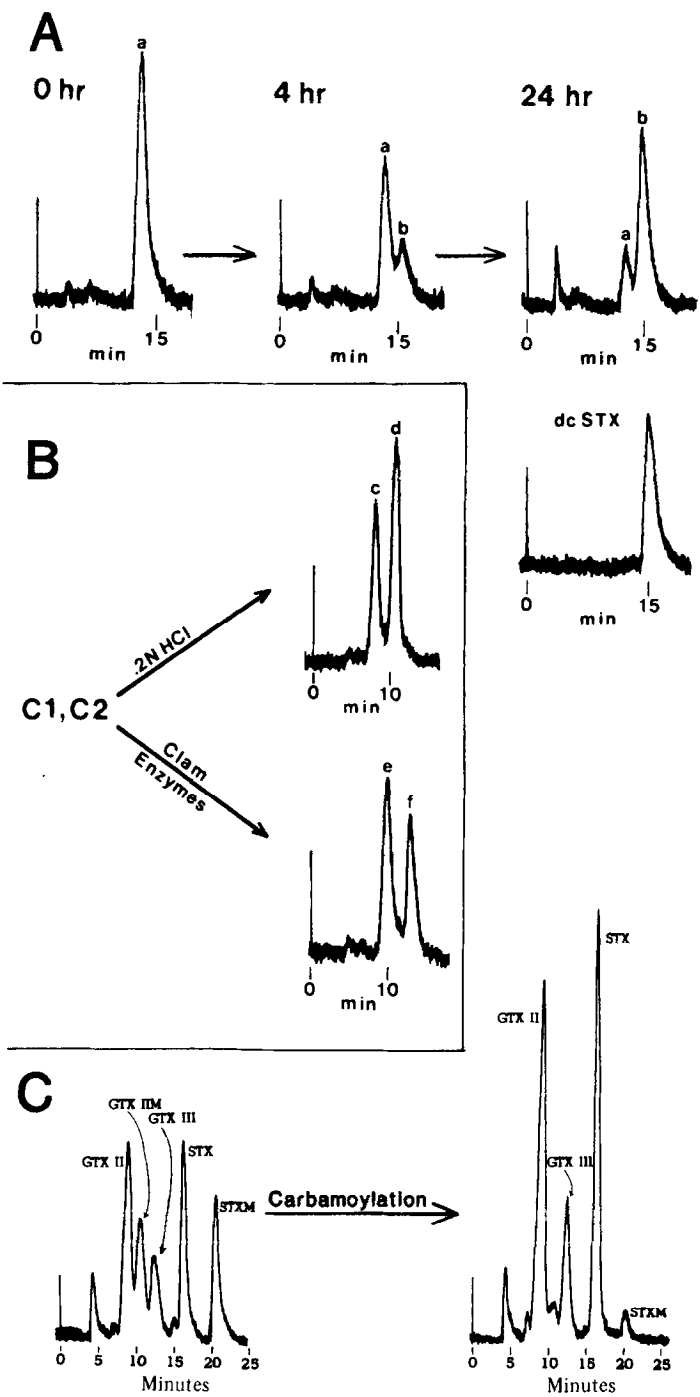


Figure 2. A: Transformation of STX (a) to STX-M (b) in homogenate of littleneck clam visceral mass (30°C, pH 6.2, STX added at the level of 15 μM). Decarbamoyl STX (dcSTX) is shown below for comparison. Incubation of B1 forms the same product (STX-M) and reaction is essentially complete after 4 hr. HPLC conditions - μ Bondapak amino column (4 mm x 30 cm) with 62% MeOH, .03M ammonium phosphate mobile phase, flow 1.2 ml/min.

B: Chromatograms of GTX-II (c), GTX-III (d), GTX-IIM (e) and GTX-IIIM (f) produced from a mixture of C1 and C2 by either acid

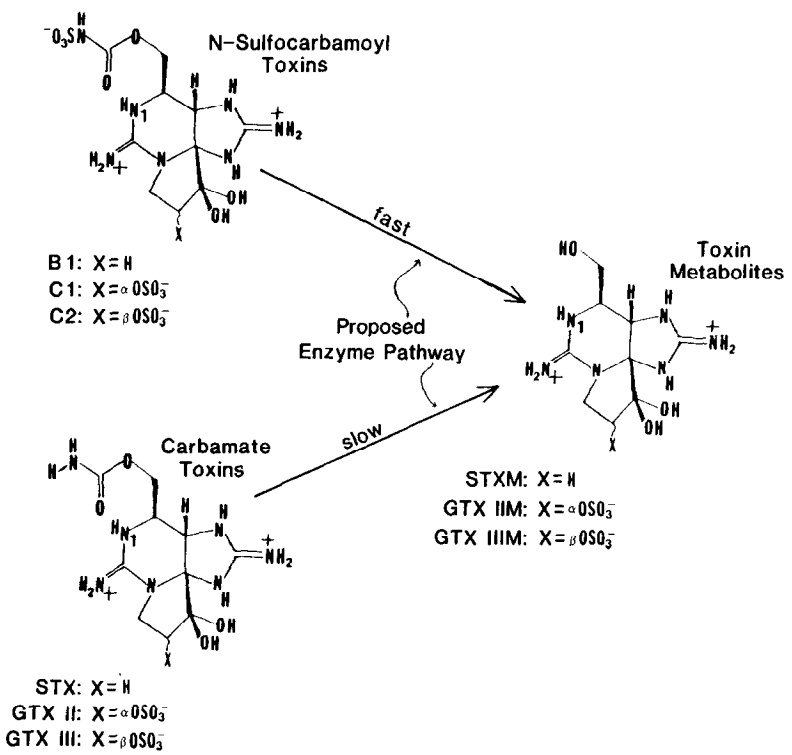


Figure 3. The metabolic transformations occurring in the littleneck clam involve hydrolysis of the carbamate group from either the N-sulfocarbamoyl or carbamate toxins (STX-saxitoxin, GTX-gonyautoxin).

important implications from a food safety standpoint since it suggests that shellfish possessing these enzymes may have a vastly different toxicity from those without the enzymes for any given total toxin content.

The observed transformations involve hydrolysis of the carbamate ester yielding the decarbamoyl derivatives (see figure 3). Support for this hypothesis includes: (a) mild acid hydrolysis (.2N HCl, 100°C) [which effectively removes the sulfo group from C1 and B1 yielding the carbamate toxins and a

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Fig. 2 Continued.

hydrolysis (.2N HCl, 100°C, 5 min) or by littleneck clam enzymes (incubation conditions as in A). HPLC conditions - Lichrosorb amino column (5 μm, 4 mm x 25 cm) with 50% MeOH, .04M ammonium phosphate mobile phase, flow 1.1 ml/min.

C: Mixture of the carbamate toxins (GTX-II/III and STX) and the metabolites (GTX-II/IIIM and STX-M) before and after carbamoylation, illustrating the conversion of GTX-II/IIIM and STX-M to GTX-II/III and STX respectively. This procedure was used to confirm that the metabolites are the decarbamoyl toxin derivatives. HPLC conditions - as in B above, except mobile phase is 60% MeOH, 0.32M ammonium phosphate, flow 0.8 ml/min.

corresponding shift in HPLC retention and toxicity] causes no change in either HPLC retention or toxicity of the metabolites, indicating the absence of a sulfo group attached at the carbamate, (b) while the toxicities of the decarbamoyl gonyautoxins are unknown[1] (due to the lack of a suitable method to hydrolyze the carbamate moiety while leaving the sulfate group (X on figure 3) intact), the toxicity of decarbamoyl saxitoxin is reportedly 1200 ± 300 MU/μmole (13), which is similar to that observed for STX-M, (c) the retention times on both the amino and cyano columns are the same for decarbamoyl STX and STX-M (see figure 2A), and (d) carbamoylation of GTX-IIM and STX-M results in the formation of GTX-II and STX respectively (as determined by their HPLC behavior; see figure 2C). Figure 3 illustrates the proposed enzymatic reaction occurring in the littleneck clams. While this transformation has only been studied using B1, C1, C2, GTX-II, GTX-III, and STX as substrates, it is likely that the enzymes involved would be active on the other reported PSP toxins.

The occurrence of the various PSP toxins in the natural environment was investigated by analyzing dinoflagellates and naturally contaminated shellfish from Puget Sound, Washington. The toxin composition in littleneck clams was found to be mostly GTX-IIM, GTX-IIIM and STX-M, suggesting that the enzymatic conversions observed *in vitro* occur *in vivo*. The toxin profile in mussels closely resembles the toxin profile of the dinoflagellates, suggesting that no metabolic transformations are occurring in these shellfish, consistent with *in vitro* laboratory observations. The toxin profile in Puget Sound dinoflagellates consists predominantly of B1, C2, GTX-III and STX, and mussels will usually contain these four toxins plus C1 and GTX-II (due possibly to epimerization of C2 and GTX-III respectively). Butter clams in Puget Sound usually contain mainly STX, which indicates that either the other toxins are being metabolically converted to STX (as has been shown in

(1) The hydrolysis of the sulfate (X in figure 3) from GTX-II, yielding 11-hydroxy saxitoxin, has been shown to have no effect on toxicity (ca 1000 MU/μmole), while the decarbamoyl 11-hydroxy saxitoxin has toxicity of 320 MU/μmole, which is very close to that found for GTX-IIM (G. Boyer, Ph.D. dissertation, U. of Wisconsin, 1980).

scallops) (11), or there is selective retention of STX in the butter clam since the dinoflagellates produce only a small quantity of STX in relation to the other toxins. In addition to saxitoxin in the butter clam, a toxin with a similar HPLC retention time to STX-M (eluting slightly late on the amino column) was often observed. Since no toxin transformations were detected in the butter clam homogenates and insufficient quantities of these samples were available to characterize the unknown peak, it was not possible to determine if it did represent STX-M. Further investigations will be necessary to determine the processes occurring in the butter clam, but it is evident that the mechanisms are very complex, involving both metabolic conversions and selective retention of some toxins in the siphon.

We have demonstrated that metabolic transformations of the PSP toxins occur in the littleneck clam and that this involves enzymatic decarbamoylation of the toxins. It is apprent from this study and those of Shimizu and Yoshioka (11) that metabolic conversions of the PSP toxins may play a very important role in determining the toxin profile and overall toxicity that develops in shellfish. These investigations also indicate that in addition to the previously reported carbamate and N-sulfocarbamoyl toxin forms, the decarbamoyl derivatives may represent a very important part of the toxin profile in some shellfish species.

The metabolic transformations occurring in the littleneck clams have important implications since the toxicities of the end products are quite different from the other PSP toxins. It is evident that the transformations result in an increase in the toxicity of the N-sulfocarbamoyl toxins and a decrease in the toxicity of the carbamate toxins by their conversion to a common end product of intermediate toxicity. Determining the presence of the decarbamoyl toxins in naturally contaminated shellfish and the species distribution of the enzymes catalyzing hydrolysis of the carbamoyl moiety are important to an understanding of the biochemical processes involved in the development of toxicity in shellfish and may partially explain the vast

differences in toxicity often observed between shellfish species during dinoflagellate blooms.

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