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Presence of paralytic shellfish poisoning toxins and soluble proteins in toxic butter clams (Saxidomus giganteus)

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Summary: Butter clams obtained from a variety of locations along the northern British Columbia coast were assayed for the presence of individual paralytic shellfish poisoning toxins (PSPT) by HPLC and total PSPT toxicity using the mouse bioassay. Specific organs, namely the siphon, adductor muscle, foot and mantle were examined for soluble antigens that crossreacted with crab Saxitoxin-Induced Protein (SIP) using immunochemical (Western blotting) techniques. Butter clams containing high concentrations of PSPT also had several proteins that crossreacted with crab anti-SIP serum. In particular, soluble proteins with distinctly different molecular weights were found in the siphon and foot, respectively, in toxic shellfish. These proteins were absent in nontoxic butter clams. The concept of using PSPT-induced proteins in the butter clam as a screen for identifying toxic shellfish is introduced.

Saxitoxin (STX) and related PSPT compounds block the sodium channels of neurons primarily in the peripheral nervous system, thereby eliciting a variety of symptoms of illness, starting with a tingling or numbness of the lips, fingertips and progressing to muscular incoordination, respiratory difficulties and in extreme cases muscular paralysis (1, 2). PSPT are found in the butter clam (Saxidomus giganteus) which acquires characteristic toxicity from feeding on the dinoflagellate Alexandrium sp.(Protogonyaulax, Gonyaulax; 3, 4). Unlike many other bivalve molluscs, the butter clam can retain PSPT for long durations without showing signs of intoxication (3, 5, 6).

Although numerous studies have shown a resistance to PSPT in various aquatic organisms such as the crab (7, 8), and bivalve mollusc (5, 9), very few data are available on the mechanism of the derived resistance to PSP toxicity. Common to both the crab and butter clam resistance to PSPT, is the observation that excitable membranes in these species are resistant to the activity of sodium channel blocking agents such as STX and tetrodotoxin (7, 9,10). In the

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butter clam, this characteristic is independent of the history of toxin contamination, or the location of the clam beds (10). Previous studies with shore crabs (Hemigrapsus oregonesis), reported finding an unique high molecular weight soluble protein, termed Saxitoxin Induced Protein (SIP), in PSPT resistant crabs, which was not present in PSPT sensitive crabs (11, 12). Recently, other workers have reported finding a novel soluble protein in the bullfrog (Rana catesbeiana) with STX binding activity and a structural dissimilarity to sodium channel proteins (13). The specific role of the soluble STX binding protein in eliciting resistance in the bullfrog to PSPT is unknown, however it is of interest that the bullfrog is more resistant to STX action than mice (14). The significance of crab SIP became more apparent with the finding that there is significant immunological crossactivity between the crab protein and unidentified soluble proteins in bivalves, such as the Pacific littleneck clam (Prototheca staminea) (15).

In the butter clam, PSPT and in particular STX are sequestered, especially in the siphon, following a period of initial uptake (6). Recent work has attempted to associate long term retention of PSPT with chemical defense against predators of the clam (10). The purpose of the present study was therefore to examine various tissues of PSPT contaminated butter clams for SIP crossreactive proteins using western blotting techniques. We propose that soluble protein present in PSPT toxic shellfish are associated with resistance to PSPT accumulation, and thus may be used to screen for butter clams containing these toxins.

MATERIALS AND METHODS

a) Butterclam Samples

The butter clams used in this study were collected between October 1988 and March, 1989 at various locations north of Prince Rupert, B.C. The specific locations are given in Table 1. Shell-fish were shucked and frozen prior to analysis of soluble protein composition, HPLC and mouse bioassay analysis of toxicity.

b) PSP Toxicity Measurements

Whole butter clam samples were analyzed for total PSPT toxicity by the mouse bioassay (8, 16). Individual PSPT (B_1, B_2, GTX 1-4, NEO and STX) were quantitated in whole butter clam homogenates by HPLC analysis. Samples were assayed 3-4 times on different days using published methods (17, 18) with a mixed PSPT standard (MS-33; 1:20 in 0.05N HoAc). The detection limits for STX, NEO and GTX II and III were 0.05 μM, 0.10 μM and 0.02 μM, respectively. Toxins were quantitated by peak area within the linear detection range for each toxin and values were corrected to μM toxin using appropriate dilutions and response factors.

c) Immunochemistry

Butter clams from the same collection batch were used for immunochemistry. The adductor muscle, siphon, mantle and foot were dissected from these bivalves on ice and soluble proteins extracted in a phosphate buffer saline (pH 7.2) at a ratio of 1:1 (w/v) prior to centrifugation at 10,000xg for 20 minutes. Clam tissue samples were adjusted to a final protein concentration of 18$\pm$ 2 mg/ml. A polyclonal antiserum to purified crab SIP (15) generated in rabbits was used in the immuno-blotting procedure (19). Specificity of the crab SIP antiserum was improved by enriching for IgG. Crude serum was fractionated with 50% saturated ammonium sulphate and the precipitated fraction passed through a sephycryl S-300 column, equilibrated in phosphate buffer (pH 6.8). The eluted IgG fraction was loaded directly onto a

DEAE cellulose column equilibrated with the same buffer. Non-binding fractions were collected and concentrated with ammonium sulphate. The resulting antiserum preparation was diluted 1:1000. Samples of butter clam tissue extracts were analyzed on 6.5% polyacrylamide gels (20) and transferred to nitrocellulose (0.45μm) paper, using a Bio-Rad mini-blotter at 100 volts for 60 min in 20mM Tris buffer containing 192mm glycine and 20% methanol (15). Blots were developed using AS-MX naphtholphosphate as the substrate in 100mM Tris-HCl (pH 7.8) buffer containing Fast Red TR salt. Molecular weights of crossreactive proteins from individual clam tissues were estimated from corresponding electrophoresis gels containing molecular weight standards (range= 45kd to 200kd; ref. 15)

RESULTS AND DISCUSSION

Butter clams with a wide range of toxicities and containing a relative mixture of individual PSPT were used in this study (Table 1). Results of the mouse bioassay were significantly correlated with the HPLC data ($p<0.001$; $r^2= 0.945$). In the majority of butter clam samples tested, STX was the principal toxin, although, neosaxitoxin (NEO) was also detected at equivalent or slightly higher concentrations in a few samples. These results agree with previous studies that have shown the butter clam to preferentially sequester STX (6, 21, 22). Previous studies have also reported an absence of enzymatic PSPT transformation in the

Table 1. Butter clam PSP toxicity monitoring by mouse bioassay, HPLC and the presence or absence of the crossreactive soluble protein (<45kd) in clam foot (SCP) [1]

Shellfish	Location	Toxicity Estimates				
		MB[2]	STX	NEO	GTX 2 and 3	SCP Sample
		(nmol/g clam tissue)				(+/-)
R3825	Canoe Pass	600	0.90	0.69	0.129	+
R3827	Canoe Pass	1100	4.22	2.99	0.203	+
R3829	Kitkatla Inlet	300	0.55	0.39	<0.02	+
R3830	Larsen Harbour	1400	3.67	2.48	0.231	+
R3831	Gasboat Pass.	520	1.03	1.09	0.166	+
R3844	Prescott Pass.	2000	6.05	7.71	0.266	+
R3852	Prescott Pass.	950	5.09	3.76	0.150	+
R3855	Finn Island	260	1.67	<0.10	<0.020	+
R2854	Humpback Bay	<80	0.84	1.42	0.074	-
Commercial Controls		<80	0.79	<0.10	<0.020	-

[1] Gasboat & Prescott Passage.
Soluble clam protein (<45kd) present in the foot (+ = present;
[2] - = absence of soluble protein).
MB= mouse bioassay (μg STX equivalents/ 100g tissue.
STX= saxitoxin; NEO= neosaxitoxin;
GTX 2 and 3 = Gonyautoxins 2 and 3.
Results represent mean of 3 to 4 determinations.

butter clam compared to other clam species (23), therefore indicating that the substantial amounts of STX and NEO, and to a lesser extent gonyautoxins (GTX), were derived from toxic dinoflagellate sources (24).

Results from immunoblotting studies using anti-SIP with PSP contaminated shellfish indicated positive crossreactivity and thus the possibility that similar PSPT induced proteins were present in PSPT contaminated butter clams (Table 1). This result confirms a preliminary report with a number of bivalve mollusc species, and in particular the Pacific littleneck clams (15). The identification of both STX as the principal PSPT with the presence of soluble protein complexes in butter clams tested herein, (Table 1) is also similar to our previous findings in the shore crab, in which an induction of soluble proteins following both the natural and artificial exposure to STX (8, 11, 12) was reported. The immunoblotting procedure was used to locate soluble clam proteins that crossreacted with crab anti-SIP in clam foot (Fig 1a), adductor muscle (Fig 1b), siphon (Fig 1c) and mantle (Fig 1d). Representative samples of toxic and nontoxic butter clams are presented in lanes 1-5. From the results of the mouse bioassay and HPLC analysis, (Table 1), it is evident that lanes 1 to 3 of immunoblots in Fig 1a-1d, represent particularly toxic clam material (R3827, R3844 and R3852, respectively), while lane 4 represents a clam sample with very low toxicity (3854). Lane 5 represents a commercial control sample with virtually no toxicity.

Unique to many of the clam organ samples tested, were several antigens that crossreacted with crab anti-SIP serum and corresponded to apparent molecular weights, derived from corresponding gels that ranged from less than 45kd to greater than 200kd. Crossreactive proteins to crab anti-SIP with apparent molecular weights greater than 150kd, in particular, appeared with different intensities in various organ samples tested, but were not related to relative PSP toxicity. In the adductor muscle,(Fig 1b), a protein band with an apparent molecular weight greater than 100kd was present to a greater extent in toxic clams than the nontoxic or a commercial clam sample. Similarly, in the siphon, (Figure 1c), proteins which corresponded to a molecular weight of less than 100kd were found to crossreact very strongly with crab anti-SIP serum in PSPT

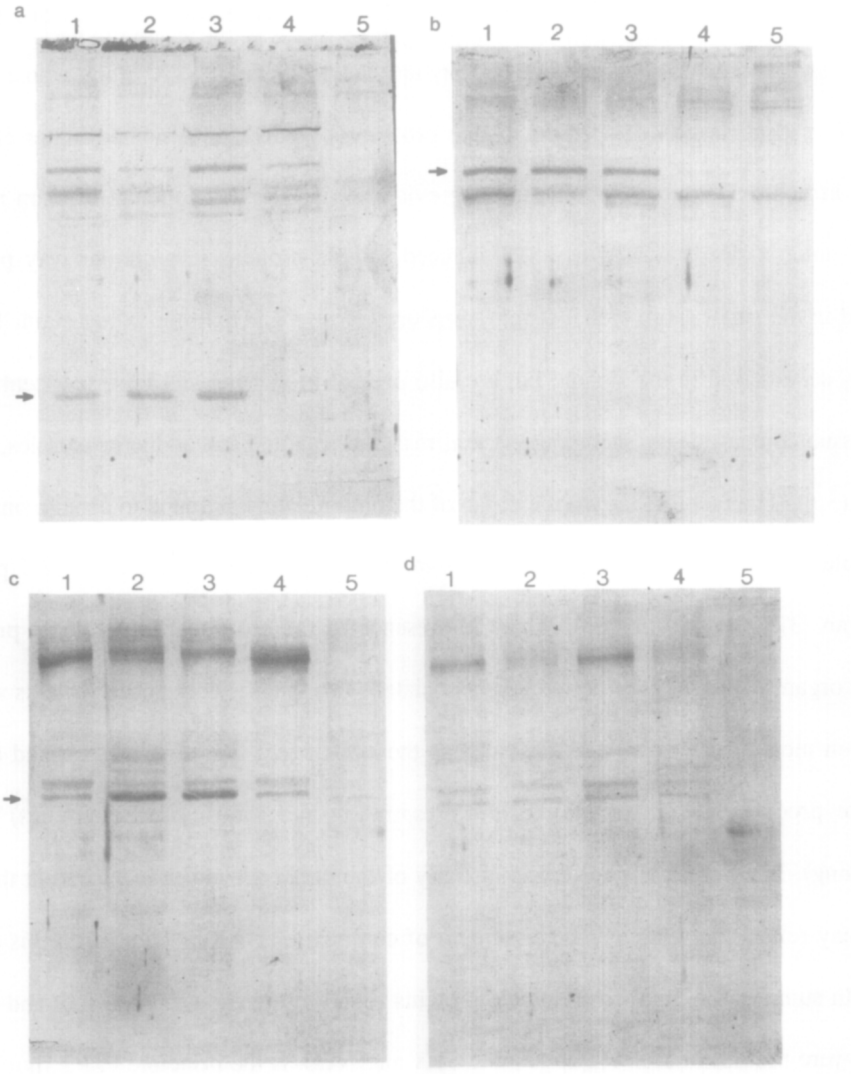

Figure 1. Representative immunoblots of crossreactive soluble proteins in clam foot (a), adductor muscle (b), siphon (c) and mantle (d). Lane 1= #R3827 (1100 μg PSP/100gm),Lane 2= #R3844 (2000 μgPSP/100gm), Lane 3=#R3852 (950 μgPSP/100gm), Lane 4= #3854 (<80 μgPSP/100gm), Lane 5=commercial control (<80 μgPSP/100gm).

toxic samples, more so than the butter clam with very low PSP toxicity (lane 3). This protein was almost completely absent in the commercial control (lane 4) sample. Mantle tissue contained in general very weak crossreactivity (Fig 1d). Finally, in the butter clam foot (Fig 1a), a distinct protein with an approximate molecular weight less than 45kd was found to crossreact strongly in PSP toxic samples, but was virtually absent in both the low toxic and commercial

control samples. The relative intensities observed in the crossreaction between this particular antigen corresponded to the total PSP toxicity and the presence of STX in the whole clam.

The identification of specific soluble proteins in PSPT contaminated butter clams, not present in nontoxic counterparts is tempting evidence that the development of clam resistance to STX autotoxicity is related to PSPT induced soluble proteins in a similar way previously reported in the small shore crab, Hemigrapsus oregonesis (8,11). In the butter clam, PSPT are not only sequestered in the siphon, but are also present in varying and lesser concentrations in other tissue compartments, including visceral mass, gill, pallial and adductor muscles, foot and gonads (3,6). The fact that the mantle organ of the butter clam was found to contain only a trace of soluble crossreactive protein, corresponds to the relatively low amount of PSPT present in this organ (3). It is also plausible, that the presence of the cross-reactive, soluble proteins in specific organ tissues may furthermore be associated with specific contractile proteins which are present in metabolically active organs such as the siphon (eg. ingestion of food and excretion of waste products), adductor muscle (e.g. respiration and shell closure activities) and foot (burrowing). In contrast, the very weak activity of the target protein(s) identified in the mantle tissue may reflect the relatively small amount of contractile activity attributed to this organ.

In summary, a number of soluble proteins found in the clam siphon, foot and adductor muscle were present in shellfish containing STX and NEO as the principle PSPT, but absent in non-toxic clam samples. The detection of these specific soluble clam proteins, in the clam foot in particular, could be used as a possible method for sceening PSPT toxic butter clams, and thus reduce the use of the mouse bioassay to PSPT confirmation testing only. Additional studies are required to characterize the tissue specific soluble proteins and to determine if PSPT-induced soluble proteins have a role in the characteristic resistance of the butter clam to PSPT.

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