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COMPARATIVE ANALYSES BY HPLC AND THE SODIUM CHANNEL AND SAXIPHILIN ^{3}H-SAXITOXIN RECEPTOR ASSAYS FOR PARALYTIC SHELLFISH TOXINS IN CRUSTACEANS AND MOLLUSCS FROM TROPICAL NORTH WEST AUSTRALIA

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Andrew Negri and Lyndon Llewellyn. Comparative analyses by HPLC and the sodium channel and saxiphilin ^{3}H-saxitoxin receptor assays for paralytic shellfish toxins in crustaceans and molluscs from tropical North West Australia. *Toxicon* **36,** 283–298, 1998.—The increased frequency and distribution of red tides requires the development of high-throughput detection methods for paralytic shellfish toxins (PST). Community ethics also requires that there be a reduced reliance upon the standard mouse bioassay. A biomolecular assay such as the sodium channel ^{3}H-saxitoxin binding assay can satisfy both of these requirements but may be compromised by cross-reactivity with the structurally unrelated tetrodotoxins (TTX). This study utilised the sodium channel assay but also an alternative ^{3}H-saxitoxin binding assay based upon a saxiphilin isoform from the centipede *Ethmostigmus rubripes* to screen for PSTs. Saxiphilin is a novel transferrin which binds saxitoxin (STX) but differs from the sodium channel in not having any measurable affinity for TTX. A detailed analysis of toxin composition was achieved by high performance liquid chromatography (HPLC). Various crustaceans and molluscs accumulate PSTs and TTX, thus proving useful biomarkers for these toxins in their immediate environment and an ideal challenge to the detection and analysis of PSTs in this presumptive screening program. Also, there has been little investigation of PSTs in invertebrates from the Indian Ocean so this region was selected to extend our knowledge of the distribution of these toxins. 190 crabs and shellfish encompassing 31 species were collected from reefs along the North-West Australian coast and tested for PSTs and TTX by sodium channel and saxiphilin bioassays as well as HPLC. PSTs were detected in 18 species of crabs and shellfish of the 31 species tested. Eight of these species have not been previously described as toxic, these being the crabs *Euzanthus exsculptus*, *Lophozozymus octodentatus*, *Metopograpsus frontalis*, *Pilumnus pulcher*, *Platypodia pseudogranulosa* and *Portunus pelagicus*, and the molluscs *Tectus fenestratus* and *Trochus hanleyanus*. By HPLC, only one or both of STX and decarbamoyl-STX was

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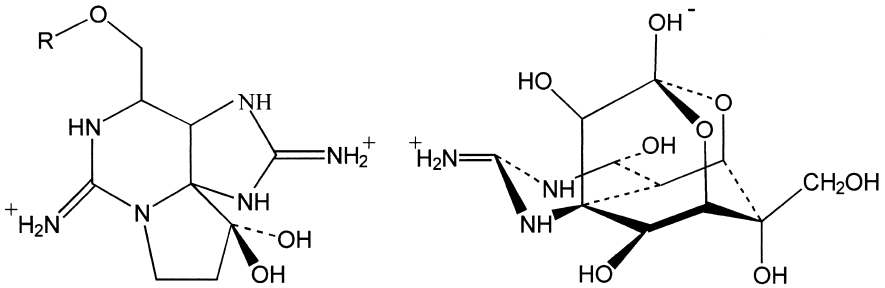
detected in any extract. Some extracts markedly inhibited ^{3}H-saxitoxin binding by the sodium channel but not by saxiphilin. The close agreement between toxin quantification by the PST specific methods of HPLC and the saxiphilin bioassay is indicative that the additional toxicity detected by the sodium channel assay is TTX.

INTRODUCTION

Human illness and fatalities due to ingestion of paralytic shellfish toxins (PST) occurs world-wide. The vectors in most of these cases have been either molluscs (Sommer and Meyer, 1937) or crabs (Hashimoto *et al.*, 1967; Noguchi *et al.*, 1969). The unpredictable toxicity of these invertebrates has been attributed to accumulation of haphazardly occurring toxins from their environment rather than innate toxin production. Thus, these animals can be considered as biofilters which magnify naturally encountered PSTs.

PSTs comprise a family of structurally related alkaloids with more than twenty variants identified. This family of toxins can be divided into three broad categories: the *N*-sulfocarbamoyl-11-hydroxysulfate C-toxins, which are the least toxic PSTs towards mammals; the singly sulfated gonyautoxins (GTX) which have a wide range of mammalian toxicities but are generally more lethal than the C-toxins; and the non-sulfated saxitoxins (STX), which are highly potent neurotoxins (Fig. 1) (Shimizu, 1988). PSTs exert their toxicity by binding to voltage-dependent sodium (Na) channels, thus blocking the influx of Na ions and preventing nerve and muscle cells from producing action potentials (Strichartz, 1986). The chemical variation of the PSTs also enables characteristic separation by high performance liquid chromatography (HPLC).

Tetrodotoxins (TTX, Fig. 1) have also been reported in a variety of marine organisms including crabs (Noguchi *et al.*, 1986; Kungsuwan *et al.*, 1988) and molluscs (Jeon *et al.*, 1984) and are sometimes reported in individuals alongside PSTs. TTXs are neurotoxins structurally unrelated to the PSTs commonly associated with puffer fish but believed to be primarily produced by bacteria (Noguchi *et al.*, 1987). It has, however,



STX R = $CONH_2$ TTX
dcSTX R = H

Fig. 1. Structures of STX, dcSTX and TTX.

been recently reported that the dinoflagellate *Alexandrium tamarense* may be the origin of TTX in some cases of paralytic shellfish poisoning (Kodama *et al.*, 1996). Despite the structural difference to the PSTs, TTX directly competes for the same binding site as PSTs on the Na channel.

Saxiphilin, a soluble circulatory protein from various vertebrates and arthropods, possesses a single, high affinity and highly specific binding site for STX and other PSTs (Mahar *et al.*, 1991; Llewellyn *et al.*, 1997). Saxiphilin is structurally unrelated to the Na channel, being instead a transferrin, proteins known primarily for their iron-binding and iron-transporting role (Morabito and Moczydlowski, 1994, 1995). Unlike the Na channel, saxiphilin does not possess any affinity for TTX making it a potentially valuable new means for specifically detecting PSTs.

The utility of a PST-specific assay based upon the affinity of radiolabelled STX to saxiphilin in a PST screening program was compared to two already validated methods, these being HPLC analysis and Na channel ^{3}H-STX binding. Unlike normal seafood safety testing programs, the more complicated HPLC analysis included all non-toxic extracts as defined by the assays to validate their sensitivity when faced with complex matrices such as crude extracts. Almost 200 individuals from four locations along the north western coastline of Australia were included in this screening program. This is a region not previously examined for the phenomenon of toxin accumulation by crustacea and molluscs. The species collected included several discovered elsewhere to be poisonous including the crab *Atergatis floridus* which is known to be capable of containing TTX alongside PSTs. This study also discovered eight species not previously reported to harbour PSTs.

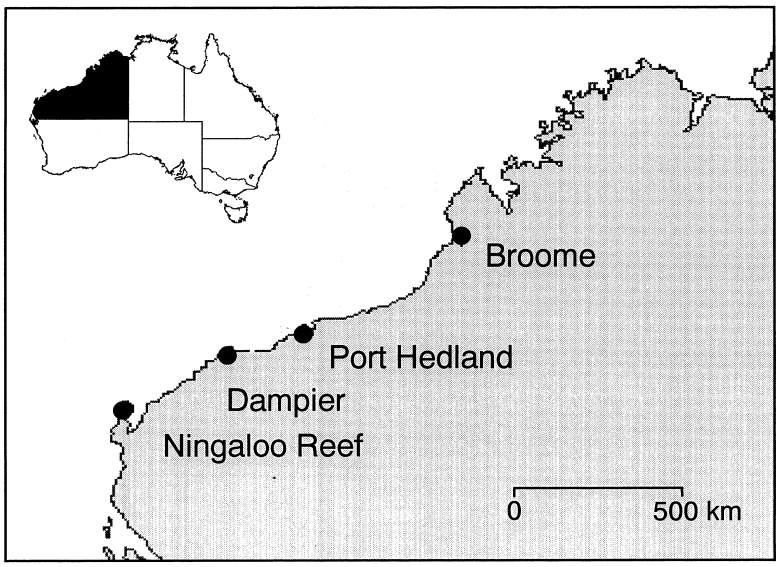


Fig. 2. Collection sites in northern Western Australia.

MATERIALS AND METHODS

Sample collection and extract preparation

All crustaceans and molluscs were collected by hand from coral reefs in the vicinity of Ninglaoo Reef (22° 58′ E, 113° 49′ S), Dampier (20° 28′ E, 116° 32′ S), Port Hedland (20° 17′ E, 118° 38′ S) and Broome (18° 00′ E, 122° 12′ S) (Fig. 2). Collections were primarily undertaken from intertidal zones at night, between October 1995 and October 1996. Organisms were anaesthetized by cooling to 4°C and stored frozen at −10°C. Individual crustaceans were blended whole while molluscs were removed from their shells before homogenising. A portion of each organism (5 g) was transferred to a vial with 5 ml of 0.1 M acetic acid. The mixture was homogenised and allowed to stand for 72 h at 4°C before centrifugation (12 000*g*, 4°C). The final extract was produced by filtration of the supernatant through a 10 000 MWCO filter (Millipore, MA).

HPLC analysis

Analyses of the three classes of PSTs (C-toxins, GTX and STX) were conducted separately according to the method of Negri and Jones (1995). Separations were performed on a Waters 600 HPLC, combined with a Pickering PCX 5100 Post-Column Reactor using a 5 μm, 250 × 4.6 mm Alltima ODS column (Alltech, IL) at a flow rate of 0.8 ml/min. Post-column oxidation followed the method of Oshima *et al*. (1993). Fluorescent PST derivatives were detected using a Linear (LC305) spectrofluorometric detector with excitation at 330 nm and emission at 390 nm. Toxins were identified by comparison of retention times and fluorescence emission spectra (λ_{max} 390 nm) with standards, kindly donated by Dr. Y. Oshima of Tohoku University, Japan and Dr. S. Hall, U.S. Food and Drug Administration, Washington DC, U.S.A. PSTs were quantified by comparison of peak areas with these same standards.

Toxin purification

A highly toxic specimen of the crab *Atergatis floridus* from Port Hedland was homogenised in the presence of 80% EtOH at pH 2 (HCl). The homogenate was centrifuged and the supernatant evaporated to dryness *in vacuo* at room temperature. The extract was re-dissolved in 0.01 M HCl and hydrophobic compounds removed with chloroform. The toxins were first chromatographed on a Bio-Gel P-2 column (Bio-Rad, CA, U.S.A.; 1.0 × 30 cm) using 0.05 N acetic acid to elute, and then with BioRex 70 (BioRad, CA, U.S.A.; 1.0 × 30 cm; H^+ form), using a gradient of 0–2 M acetic acid. The STX fraction from this column was rechromatographed on the BioRex 70 column to obtain pure STX.

Biomolecular assays

All buffers and general chemicals were from Sigma (St. Louis, MO) and water deionised (> 18 MΩ) with a Barnstead system (Barnstead, IA, U.S.A.). Rat brain membranes containing intact Na channels were prepared as in Llewellyn (1997) from postmortem rat brain tissue kindly provided by the Department of Molecular Sciences, James Cook University of North Queensland, Townsville, Australia. Rats were handled and sacrificed according to the ethics guidelines of this university. As a source of saxiphilin, haemolymph was obtained from hand-collected tropical centipedes, *Ethmostigmus rubripes*, after sacrificing animals anaesthetized by hypothermia. Protease inhibitors were added to the haemolymph and cellular material removed by filtration and centrifugation as for crustacean saxiphilin (Llewellyn, 1997). Both rat brain membranes and centipede haemolymph were stored at −80°C.

^{3}H-STX (Amersham, UK) binding by rat brain synaptosomes (300 μg protein/ml) was measured as described in Llewellyn (1997) using standard assay conditions of 20 mM Mops-NaOH (pH 7.4), 200 mM choline chloride, 0.1 mM EDTA, 2 nM [^{3}H]STX (Amersham, UK). Binding assays with haemolymph from *E. rubripes* were conducted as for rat brain synaptosomes except for a lower protein concentration (180 μg protein/ml), and replacement of the choline chloride with 200 mM NaCl. Every experiment included one sample containing no competitor or extract to measure total ^{3}H-STX binding, while another sample contained 10 μM unlabelled STX (Calbiochem, CA, U.S.A.) to determine non-specific binding. In all experiments, these values were used to normalise data from test samples to 100%.

Samples were screened for inhibition of ^{3}H-STX binding by incubating 10 μl of each crab and molluscan extract in standard assay conditions. Extracts which inhibited ^{3}H-STX binding by either the Na channel and centipede haemolymph by more than 3 standard deviations from the mean of the screening results (see Section 3 for explanation), were quantified by titration in a competition experiment using standard assay conditions for the respective STX-receptor. All competition curves were fit with the equation: $f = 100/(100 + [\text{competitor}]/IC_{50}^{n})$; where f is the normalised level of bound ^{3}H-STX in per cent, IC_{50} is the volume of crude extract which caused 50% inhibition, and n is the slope of the curve. Since the concentration of the ^{3}H-STX used in the experiments was significantly greater than the K_D of the receptors for the radiolabelled toxin, the IC_{50} of unlabelled STX vs ^{3}H-STX should be equivalent to the concentration of ^{3}H-STX used (Cheng and Prusoff, 1973). This was confirmed in two competition experiments using standard assay conditions which obtained IC_{50}'s of 1.8 ± 0.2 and 1.7 ± 0.3 nM unlabelled STX for the Na channel and saxiphilin, respectively (data not shown), values which closely approximate the concentration of ^{3}H-STX used (2 nM). In both cases, the slope

of the competition curve was one. This property was used to calculate the equivalent STX concentration [STXeq] in nanomolar of crude extracts with the equation: [STXeq] = $500/IC_{50}$, using the IC_{50} obtained from competition curves described above. The factor of 500 takes into account that the extract was competing against 2 nM ^{3}H-STX and the assay sample volume (i.e. 250 μl). The stock nM value for STXeq was then converted to μg/100 g from the original weight of tissue extracted and extract volumes.

RESULTS

Bioassay and HPLC screening

The majority of the histograms of inhibition values from screening the 190 extracts in both the Na channel and saxiphilin assays fell under the simple Gaussian distribution: $(k/s)*e^{[-0.5\{(x-m)/s\}^2]}$ (Fig. 3), where k is a weighting factor, s is the standard deviation and m is the arithmetic mean. The respective distributions, expressed as per cent of control, for the Na channel and saxiphilin screening results were curves about the means of 71.8 and 85.9% with standard deviations of 12.3 and 17.3%. Fitting the histograms with a two population Gaussian distribution did not alter these values for the major histogram peaks. From these normal distributions, extracts defined as toxic were those which caused inhibition greater than 3 standard deviations from the mean and could therefore be statistically considered significantly different from the mean (i.e. $p < 0.01$), and warranted quantitation by competition curves. Any extract therefore, which reduced the bound ^{3}H-STX to less than 34.9 and 34% relative to control samples in the Na channel and saxiphilin screening assays, respectively, were considered to contain significant inhibitory activity. This meant that 46 (44 crustaceans, 2 molluscs) and 29 (28 crustaceans, 1 mollusc) extracts were deemed toxic from Na channel and saxiphilin

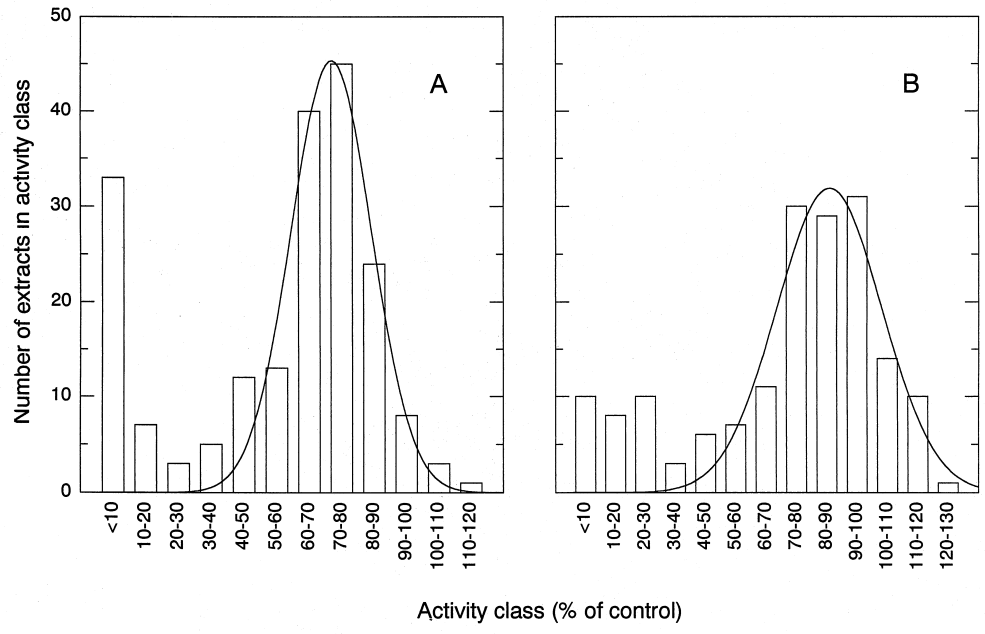


Fig. 3. Histograms of inhibition values in the Na channel (A) and Saxiphilin (B) assays overlaid with the Gaussian distributions described in the text.

Table 1. Toxin concentration of individual crustacean and molluscan extracts defined as toxic by at least one bioassay

Species	Site*	Individual‡	dcSTX HPLC	STX HPLC	Total PST HPLC	STXeq† Na channel	STXeq saxiphilin
Crustaceans							
Atergatis floridus	NR	1	7.0	47	54	262¶	–
		2	12	2.6	14	464¶	7.3
		3	15	2.8	18	1718¶	13
		4	40	4.7	44	431¶	–
		5	21	42	63	459¶	36
	DA	6	32	13	46	305¶	–
		7	16	106	122¶	155¶	344¶
		8(□)	60	13	73	54	–
		9	3.2	28	31	214¶	74
		10	45	24	69	64	13
		11	3.6	15	18	233¶	8.6
		12	36	0.0	36	222¶	–
		13	22.0	0.0	22	174¶	2.8
		14	18	13	32	13	–
		15	3.3	3.0	6.3	124¶	–
		16	0.0	44	44	41	76
	PH	17	836	5391	6227¶	16611¶	5772¶
		18	57	388	445¶	489¶	340¶
		19	61.1	2.5	63	2121¶	23
		20	8.6	350	359¶	1680¶	616¶
Euzanthus exsculptus	PH	21	6.2	22	29	17	29
		22(◇)	12	55	67	52	44
Lophozozymus octodentatus	BR	23	19	3.7	23	7.5	7.9
	PH	24	4.0	5.2	9.2	4.6	5.2
		25	64	10	74	24	29
Neoxanthias impressus	NR	26	17	0.0	17	147¶	–
		27	0.0	3.6	3.6	459¶	–
Pilumnus pulcher	PH	28	0.0	15	15	22	22
		29(×)	0.0	29	29	33	32
		30(○)	11	24	34	80¶	32
		31	0.0	17	17	14	15
Pilumnus vespertilio	NR	32	0.0	0.0	0.0	1.7	–
	PH	33	0.0	12	12	57	30
		34	0.0	74	74	83¶	94¶
		35	0.0	96	96¶	54	120¶
		36(△)	0.0	67	67	49	64
Platypodia pseudogranulosa	DA	37	7.8	2.5	10	3.4	3.6
		38	51	0.0	51	7.0	12
Zosimus aeneus	NR	39(⊕)	28	13	41	17	–
		40	5.3	2.2	7.5	5.0	–
		41	12	9.8	22	36	–
		42(⬡)	26	9.9	36	16	–
		43	13	8.3	22	10	–
		44(∇)	30	7.0	37	78	–
Molluscs							
Tectus fenestratus	DA	45	0.0	7.5	7.5	3.6	–
Turbo argyrostomus	DA	46	13	0.0	13	12	14

*NR, Ningaloo Reef; DA, Dampier; PH, Port Hedland; BR, Broome.
–Indicates non-significant inhibitory activity when screened and did not warrant bioassay quantification.
‡The number or symbol of each individual corresponds to each sample plotted in Fig. 7(a–c).
†STX equivalents calculated as described in Section 2 and equated to μg/100 g organism.
¶Individuals containing >80 μg/100 g tissue and thus dangerous for human consumption.

screening results and were quantified by competition experiments (Table 1). At this stage, a disparity between the two assays was apparent where several extracts significantly inhibited ^{3}H-STX binding to the Na channel yet had little effect upon saxiphilin binding of ^{3}H-STX. For example, an individual of *Neoxanthias impressus* (# 27 in Table 1) was quite active in the Na channel assay but did not cause significant inhibition when screened in the saxiphilin assay.

From the lowest values in Tables 1 and 2, the detection limits for the biomolecular assays and HPLC can be estimated to be 2–3 μg STXeq/100 g organism. The reliability of the bioassays near this limit is reduced though, as there were some false negatives with 22 extracts (17 crustaceans, 5 molluscs) not being defined as toxic by the criteria used in this study but were later found to have trace amounts of PSTs upon HPLC analysis (Table 2). Extending the above method of defining toxic extracts to include those extracts having caused inhibition between 2 and 3 standard deviations from the mean (i.e. $p < 0.05$) of the screening histograms did not redefine many of these non-toxic extracts as toxic (Table 2). In only one case (#32) was significant Na channel binding activity observed when HPLC detected no toxins.

Table 2. Levels of HPLC identified PSTs in extracts not defined as toxic after screening in the bioassays

Species	Site*	dcSTX[†]	STX[†]	STX eq[†]
Crustaceans				
Carpilius maculatus	NR	6.7	0.0	6.7
Eriphia sebana	NR	2.1	1.4	3.5
		10.3	0.5	10.8
		4.1	0.3	4.4
Lophozozymus octodentatus	PH	12.2	1.6	13.8
		8.2	1.9	10.1[¶]
Metopograpsus frontalis	BR	10.0	0.0	10.0
Pilodius areolatus	NR	6.9	5.2	12.1[¶]
		26.0	3.0	29.0
Pilumnus vespertilio	NR	0.0	2.6	2.6
		0.0	2.0	2.0[§]
		0.0	1.4	1.4[¶]
	BR	38.3	3.1	41.4
		6.1	2.5	8.6
		9.9	3.3	13.2
	DA	0.0	12.3	12.3
Portunus pelagicus	PH	0.0	1.8	1.8
Molluscs				
Tectus fenestratus	DA	6.8	5.9	12.7[¶]
		12.1	6.6	18.7
Tectus niloticus	BR	13.0	0.0	13.0[¶,§]
Tectus pyramis	DA	0.0	16.6	16.6
Trochus hanleyanus	DA	4.8	0.0	4.8

*NR, Ningaloo Reef; DA, Dampier; PH, Port Hedland; BR, Broome.

[†]Toxicity expressed as μg/100 g organism.

[¶]Inhibition value when screening extract with the Na channel was >2 standard deviations from the mean of the histogram.

[§]Inhibition value when screening extract with the saxiphilin was >2 standard deviations from the mean of the histogram.

Toxin quantification by competition curves and HPLC

The phenomenon first observed after the bioassay screening where the two assays detected markedly different levels of inhibition by the same extract became fully apparent during quantification of toxicity by competition curves. The IC_{50}'s for several extracts in the Na channel assay were much lower than the IC_{50} for the same extract in the saxiphilin assay. This is most evident when comparing STXeq values from the bioassays for extracts from *A. floridus* (Table 1) with there being up to a 130-fold difference between the values obtained by the two assays. This contrast in toxin levels detected by each assay is clearly illustrated by the combined Na channel and saxiphilin competition curves for *A. floridus* (#13 in Table 1) and *Pilumnus vespertilio* (#34 in Table 1) (Fig. 4). The inhibition curves in both bioassays for the extracts from *P. vespertilio* were virtually identical whereas there was almost 100 fold more Na channel blocking activity in the *A. floridus* extract than can be explained by the PSTs detected using the saxiphilin assay. HPLC analysis of these two extracts reinforced this pattern with the same value obtained for PSTs in *P. vespertilio* and less agreement between both biomolecular assays and HPLC toxin quantification in the *A. floridus* extract.

The competition curves for all of the extracts using saxiphilin as the STX-receptor and virtually all of those quantified with the Na-channel binding assay were consistent with inhibition at a single binding site by a single component and thus well fit by the standard competition curve described in Section 2. In these instances, the slopes of the inhibition curves approximated unity and ranged from 0.8 to 1.2. The largest error when fitting these competition curves was 17%. The exceptions in the Na channel competition data, where the data was poorly fit with the single site competition curve, is exemplified in Fig. 5 with the extract from *A. floridus*. A significantly better fit ($p \ll 0.01$) to this data, as measured with an *F*-statistic calculated by the extra sum-of-squares

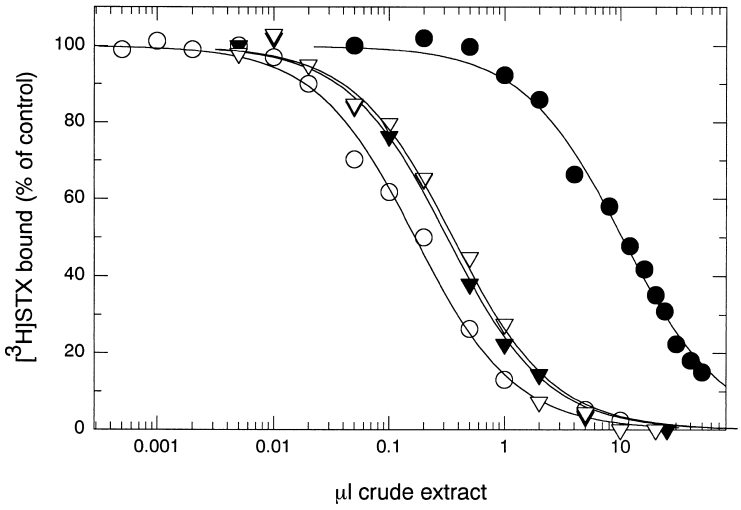


Fig. 4. Competition curves using both the Na channel and saxiphilin receptor assays obtained by titrating extracts of individuals 13 and 34 in Table 1. Extracts from *A. floridus* (individual 13 in Table 1; circles) and *P. vespertilio* (individual 34 in Table 1; triangles) were titrated in the presence of Na channel (hollow symbols) and saxiphilin (filled symbols). Curves shown are those from fitting with the single site competition curve described in Section 2.

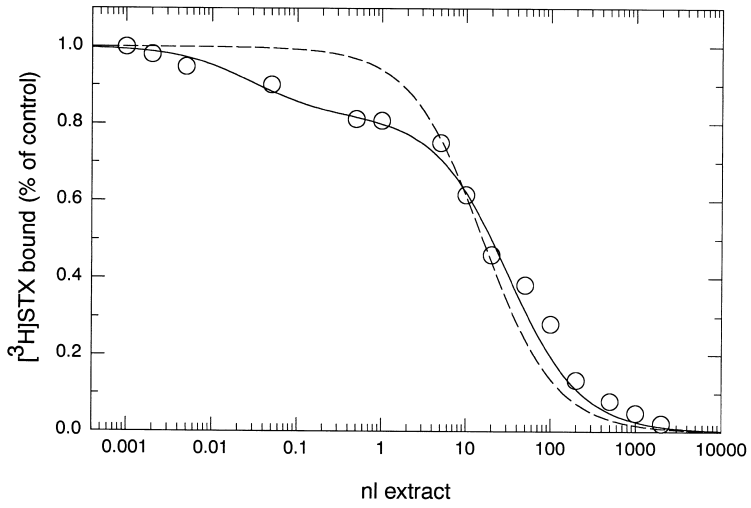


Fig. 5. Competition curve for extract from a specimen of *Atergatis floridus* (individual 3) using the Na channel assay. The data obtained after titrating the extract in the presence of Na channels in standard assay conditions was better fit with a two site competition curve (solid line) than if a single site competition curve was used (dashed line).

method described in Draper and Smith (1966), was obtained with a competition curve assuming two binding sites: $f = \{a/(100 + [\text{competitor}]/\text{IC}'^{n}_{50})\} + \{(100 - a)/(100 + [\text{competitor}]/\text{IC}''^{n}_{50})\}$ (Doyle *et al.*, 1993); where the additional parameter a is the percentage of the total number of binding sites belonging to each component of the curve. In this particular instance, the values obtained were $\text{IC}_{50'} = 531$ nl and $\text{IC}_{50''} = 1.4$ nl, $n = 1.1$ and $a = 18$.

Toxin quantification with the Na channel assay correlated poorly ($r^2 = 0.33$) with HPLC derived values for the same extracts [Fig. 6(a)]. Many of the data points were skewed away from the 1:1 relationship where toxin levels calculated from the Na channel assay and HPLC are equivalent, with the Na channel assay detecting higher toxin levels than HPLC. Most of the extracts biassed towards the Na channel assay were from *A. floridus* [see individuals 2, 3, 5, 11 and 19 in Table 1 and Fig. 6(a)]. An improved correlation ($r^2 = 0.81$) was obtained when comparing toxicity levels derived from saxiphilin competition curves and HPLC analysis [Fig. 6(b)]. The activity of many of the *A. floridus* extracts were attenuated in the PST-specific saxiphilin assay, with similar STXeq levels to those measured by HPLC.

Toxin composition as measured by HPLC

HPLC analysis revealed a simple toxin profile for each of the toxic extracts, with varying combinations of STX and dcSTX (Fig. 7, Tables 1 and 2). Sulfated and sulfonated analogues of STX such as the GTXs or C-toxins were not detected in any of the extracts. Fluorescence emission spectra of HPLC peaks were compared with the spectra of standard PSTs in each case except for those extracts in Table 2 which contained toxin levels too low for confirmation by this means and the toxins are identified solely by comparison of retention times to standards. Several peaks eluting at similar retention

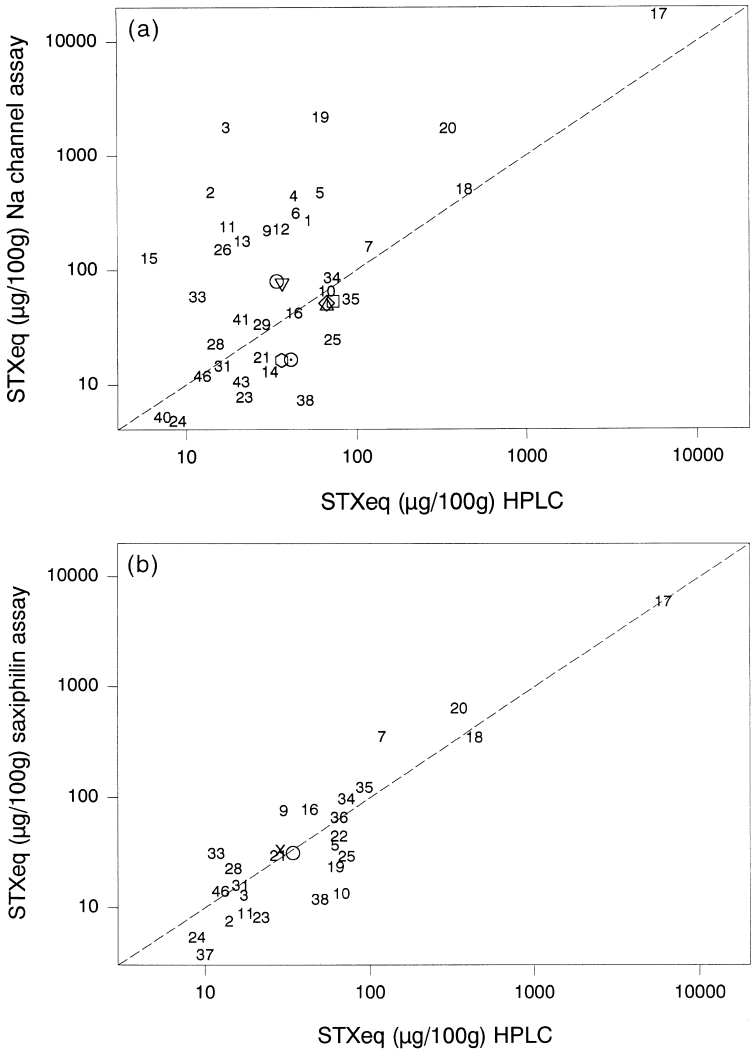


Fig. 6. Correlation of the total toxin concentration of crustacean and mollusc extracts expressed as saxitoxin equivalents (STXeq) as calculated by HPLC and the two binding assays: (A) HPLC vs Na channel assay and (B) HPLC vs saxiphilin assay. The IC_{50}'s for dcSTX vs 2 nM ^{3}H-STX of 6.4 nM and 1.8 nM for the Na channel and saxiphilin assays, respectively, (data not shown) were used to convert the concentration of dcSTX as calculated by HPLC to STXeq for direct comparison with assay data (i.e. reducing factors of 3.2 and 0.9, respectively). The dotted line in each case represents an ideal 1:1 relationship where activities in the assays are equal or explained fully by the HPLC data. The numbers and symbols graphed represent data points and correspond to samples in Table 1.

times to GTXs were detected but these peaks did not exhibit the characteristic PST emission maxima at 390 nm. Standard curves were reproduced periodically during these analyses and repeated injections of standards never varied by more than 10%.

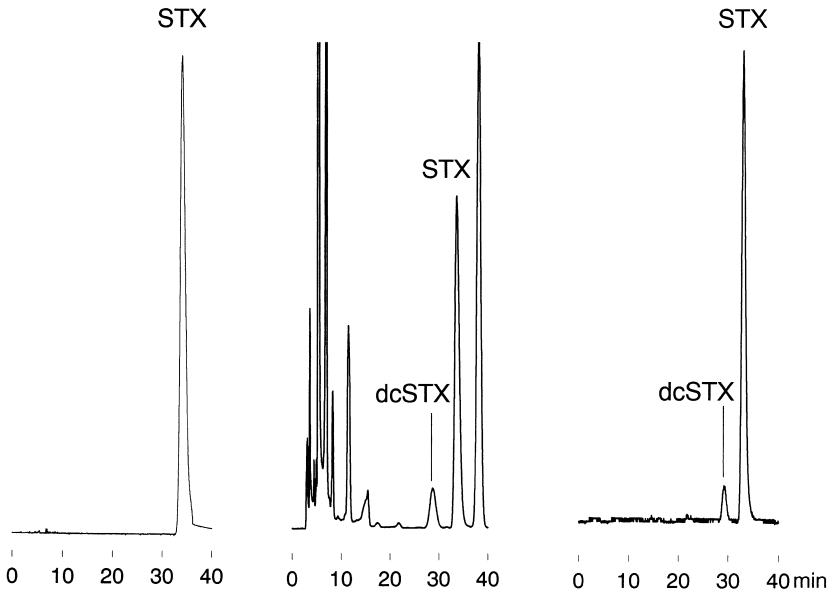


Fig. 7. Chromatograms of STX standard (A), extract from the crab *A. floridus* (B; No. 17) and dcSTX and STX isolated from *A. floridus* (C; No. 17). Note that fluorescence emission intensity scales are not equivalent between chromatograms.

HPLC of *A. floridus* (#17) fractions (BioGel P-2 and BioRex 70) did not reveal C-toxins or GTXs possibly hidden by interfering peaks. Fluorescence emission spectra confirmed the presence of STX and dcSTX in both the raw extracts of *A. floridus* and other crustaceans and molluscs as well as in the purified *A. floridus* fractions ($\Lambda_{\text{max, emission}} = 390$ nm).

Taxonomic and biogeographic patterns of toxicity

PSTs were detected in 8 species where toxins had not previously been recorded, these being the crustaceans *Euzanthus exsculptus*, *Lophozozymus octodentatus*, *Pilumnus pulcher*, *Platypodia pseudogranulosa* (Family Xanthidae), *Metopograpsus frontalis* (Family Grapsidae) and *Portunus pelagicus* (Family Portunidae); and the prosobranch molluscs *Tectus fenestratus* and *Trochus hanleyanus* (Family Trochidae). This list includes one commercial species of crustacean, the Blue Manna Crab (*P. pelagicus*) which contained low levels of STX at the Port Hedland site (Table 2).

PSTs were not detected in the crustaceans *Grapsus albolineatus*, *Metopograpsus thukuhar* (Family Grapsidae), *Micippa platipes* (Fam. Majidae), *Ocypode ceratophthalma*, *O. convexa* (Family Ocypodidae), *Charybdis helleri*, *Thalamita stimpsoni* (Family Portunidae), *Cymo* sp., *Myomenippe formasinii*, *Neoxanthias impressus*, *Xanthias lamarcki* (Family Xanthidae), the polyplacophoran mollusc *Acanthopleura gemmata*, or the prosobranch molluscs *Angaria tyria*, *Turbo laminiferus* (Fam. Turbinidae), *Conus lividus* (Fam. Conidae), *Thais aculeata*, *Morula granulata* (Fam. Muricidae), *Nerita plicata*, *N. undata* (Fam. Neritidae), *Pinctada margaritifera* (Fam. Pteriidae), and *Septifer bilocularis* (Fam. Mytilidae).

Of the four collection sites, Port Hedland had the most toxic organisms with HPLC detecting PSTs in 19 of the 20 individuals from the 6 species collected including 4 newly toxic species of crabs. The only non-toxic specimen was an individual of the commercial crab species *P. pelagicus*. This contrasted with the highly diverse collection from the Ningaloo Reef, a comparatively pristine National Park several hundred kilometres south of Port Hedland, which yielded 114 individuals from 27 species, of which only 23 (7 species) contained HPLC detectable levels of PST. Twenty of the 41 individuals collected at Dampier were toxic as were 6 individuals of the 15 crustaceans and molluscs collected at Broome. Individuals from Port Hedland also exhibited the highest toxicity, with 4 of the 5 most toxic samples (by HPLC) coming from this site (Table 1). Also, while the crab *A. floridus* was toxic at all sites, this species was most toxic at Port Hedland (Table 1). Another example of the higher level and incidence of toxicity at Port Hedland is the crabs *Pilumnus vespertilio* and *P. pulcher* with all specimens from this site being toxic which was rarely the case at Dampier, the only other site where they were collected.

DISCUSSION

Biomolecular assays have a sample capacity and throughput unmatched by most other analytical methods. This is most dramatically demonstrated in drug discovery screening programs where some pharmaceutical companies have the capacity to test 100 000 samples per day (Dutton, 1997). Such sample capacity is beyond the requirements of seafood toxin testing laboratories but progress is being made towards higher throughput biomolecular PST assays, which capitalise upon the affinity of the Na channel for these toxins (Doucette *et al.*, 1997). An alternative PST receptor is saxiphilin which differs from the Na channel in being hydrophilic and TTX insensitive, with the one isoform cloned to date showing saxiphilin to be a transferrin (Morabito and Moczydlowski, 1994, 1995). The Australian centipede *Ethmostigmus rubripes* possesses a saxiphilin-like activity with a very high affinity for STX (< 1 pM) and similar affinities for dcSTX, neosaxitoxin (NEO) and B1 (Llewellyn *et al.*, 1997). This high affinity and relative non-selective binding of different PSTs, coupled with the insensitivity for TTX, make centipede saxiphilin a promising candidate for development as a PST-specific receptor assay to parallel the Na channel bioassay. Its failing is that, like the Na channel (Strichartz, 1986), it binds a representative of the C-toxins, C1, with much lower affinity (Llewellyn *et al.*, 1997). In light of the known pharmacological properties of centipede saxiphilin and the Na channel, the dual bioassay approach adopted here allowed an early hypothesis to be generated after the initial screening that TTX-like compounds were present in some extracts which caused marked inhibition of ^{3}H-STX binding by the Na channel but not by saxiphilin. The difference in responsiveness of the two assays to inhibitory activity was accentuated when extracts were quantified with competition curves.

Determining the significance of a level of activity in a bioassay screening program is often arbitrary. Fast assays allow rapid dataset creation which can be analysed to obtain an objective means of defining toxicity. In our case, this was achieved statistically by simply fitting the screening data histogram to a Gaussian curve. This was a successful approach as all but one of the extracts defined as toxic by this means were found to contain PSTs by HPLC. At the detection limit of the bioassays, some unreliability occurred as a number of extracts with trace amounts of PSTs were not detected by

either bioassay in the initial screen, including some which were above the putative detection limit of the assays. A number of extracts which were not defined as toxic by the saxiphilin assay contained PSTs as detected by HPLC, but a majority of the toxicity in these extracts could be attributed to non-PST Na channel activity.

The challenge to bioassays by complex biological samples containing more than a single toxin is the governance of the ligand–receptor interaction by the law of mass action. Several examples were encountered here where an extract produced a bimodal competition curve. In these instances, the more complex inhibition curves would most likely be attributed to resolution of a mixture of agents with different affinities at suitable, relative concentrations to allow the phenomenon to be observed. HPLC analysis of the extract in Fig. 5 (individual #3 in Table 1) where this occurred revealed that dcSTX and STX each contributed 16 and 84% to the total PST suite, respectively. HPLC analysis in combination with the saxiphilin result also revealed that there was 1700 μg/100 g of unexplained, non-PST Na channel activity in this extract. Variation of toxin affinities by the biological receptors could jeopardize the utility of biomolecular assays by not detecting minor, but significant, amounts of highly active toxins which are overwhelmed in an assay by larger concentrations of toxins with lower efficacy. A mixture of toxins will more likely mimic a homogeneous population of compounds when binding affinities are most alike and thus the less selective the binding site used in a biomolecular assay, the more such assays will approach the objectivity of chemical analysis.

The downfall of biomolecular assays is that they cannot identify individual components within a toxic extract. At present, HPLC analysis is the most reliable technique to achieve this end but it is a slow process and not suitable for high sample throughput. Two or three chromatographic analyses are required for each sample as slightly different conditions are necessary to separate the three PST classes (C-toxins, GTXs and STXs). The HPLC analytical system adopted here required three analyses which resulted in the discovery of only STX and dcSTX in any of the extracts from tropical Western Australian organisms. In several instances, the Na channel assay detected higher levels of activity than could be explained by the amounts of STX or dcSTX detected by HPLC or total toxicity from the saxiphilin assay. For example, the Na channel assay detected 1718 μg STXeq/100 g in extract No. 3, almost 100 fold greater than the level calculated from the saxiphilin assay or HPLC. When toxicity calculated from the Na channel binding assays was plotted against the HPLC results, many of the extracts were biased towards high Na channel activity, resulting in a poor correlation. It is likely that the unknown Na channel inhibitors are TTX or TTX analogues, which compete with STX for the same site on the Na channel but have no effect upon saxiphilin binding of ^{3}H-STX. The correlation between HPLC and saxiphilin data was significantly better which reflects the high specificity towards PSTs of both techniques. The most striking cases of unexplained Na channel activity were extracts 2, 3, 9, 11, 17 and 19. Each of these extracts were from *A. floridus*, a species of crab in which both PSTs and TTXs have previously been detected in the same individual (Noguchi *et al.*, 1983). Preliminary HPLC results have identified TTX in several of these extracts based upon comparison of retention times, spiking experiments and identical behaviour to TTX standards when manipulating post-column derivatisation (data not shown).

The simple toxin profile of various combinations of STX and dcSTX in all animals examined from Western Australia contrasts with the occurrence of STX and the hydroxylated and sulfonated STX analogues, NEO, GTX_1 and GTX_2 detected in crabs from the Great Barrier Reef (Llewellyn and Endean, 1991). For further comparison,

STX, NEO, dcSTX, GTX_1 and GTX_2 co-occurred in several individual crustaceans from tropical Fiji (Raj *et al*., 1983). The molluscan toxin profile also contrasted with the more complex profile of closely related gastropods collected in Japanese waters (*Turbo marmorata*, *Turbo argrostomata* and *Tectus pyramis*) which contained GTX_2, GTX_3, neoSTX, dcSTX and STX (Oshima *et al*., 1984). The almost uniform toxin profiles in Western Australian crustaceans and molluscs and the consistency between species and sites is suggestive of a similar, or even common, source of toxins. It is possible however, that the primary toxin producers exhibit entirely different toxin profiles, as STX and dcSTX are the final detectable degradation products of the various C-toxins and GTXs which undergo chemical and enzymatically mediated reductive cleavage (desulfation and dehydroxylation) and hydrolysis (decarbomoylation and desulfation) (Sullivan *et al*., 1983; Oshima *et al*., 1984; Oshima, 1993; Cembella *et al*., 1993; Jones and Negri, 1997).

The maximum toxin level detected in this survey was over 16 000 μg STXeq/100 g in a specimen of *A. floridus* according to the Na channel assay which is similar to maximum reported PST levels in *A. floridus* from Fiji of 12 900 μg STXeq/100 g (Raj *et al*., 1983). This level far surpasses the highest level of 1944 μg/100 g recorded from *A. floridus* on the Great Barrier Reef of Eastern Australia (Llewellyn and Endean, 1991). The majority of samples in this survey however, contained less than 80 μg PST/100 g, the level accepted as safe for human consumption (van Egmond *et al*., 1991). The toxicity levels for crabs collected in Australia have yet to reach the massive amounts reported for crustaceans from coastal Asian waters. In one report, an individual of *Zosimus aeneus* was found in Japan to contain nearly 16 500 MU/g (Koyama *et al*., 1983) which equates to 300 000 μg STXeq/100 g tissue (1 MU = 0.18 μg STX; Schantz *et al*., 1957).

Xanthid crabs remain by far the most commonly toxic crustaceans, and this study extends the list of potentially toxic species (*Euzanthus exsculptus*, *Lophozozymus octodentatus*, *Platypodia pseudogranulosa*). Other crustacean families do accumulate toxins but never to the levels attained within some xanthid individuals. The recent report of a putative PST resistance mechanism in certain xanthids by a haemolymph protein pharmacologically similar to saxiphilin and its absence within regularly toxic species such as *A. floridus* may be evidence of natural selection for certain xanthids to accumulate PSTs (Llewellyn, 1997). Other families of crabs always have low amounts of toxins and the few toxic representatives collected may simply be tolerant individuals containing sublethal amounts of toxins or intoxicated and affected individuals which have yet to die.

Until the past decade, PSTs were solely detected by injection of mice, with the signs exhibited by intoxicated mice used to confirm toxin identity and the time to death being the quantitative measure of toxicity. This practice is decreasing as scientists pursue more humane testing methods such as receptor assays (Doucette *et al*., 1997) which include cell based bioassays (Jellett *et al*., 1992), ELISA assays (Usleber *et al*., 1991) and chemical detection (Oshima, 1993). The battery of tools available to this study, namely the PST and TTX sensitive Na channel assay, a PST specific saxiphilin bioassay and HPLC toxin analysis provides an illuminating approach even from the earliest stage of bioassay screening in a PST testing program.

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