SPECIAL GUEST EDITOR SECTION

Integrated Enzyme-Linked Immunosorbent Assay Screening System for Amnesic, Neurotoxic, Diarrhetic, and Paralytic Shellfish Poisoning Toxins Found in New Zealand

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Enzyme-linked immunosorbent assays (ELISAs) were developed for amnesic, neurotoxic, and diarrhetic shellfish poisoning (ASP, NSP, and DSP) toxins and for yessotoxin. These assays, along with a commercially available paralytic shellfish poisoning (PSP) ELISA, were used to test the feasibility of an ELISA-based screening system. It was concluded that such a system to identify suspect shellfish samples, for subsequent analysis by methods approved by international regulatory authorities, is feasible. The assays had sufficient sensitivity and can be used on simple shellfish extracts. Alcohol extraction gave good recovery of all toxin groups. The ease of ELISAs permits the ready expansion of the system to screen for other toxins, as new ELISAs become available.

Regulatory monitoring of shellfish for phycotoxins in New Zealand is now well established, and a well coordinated research effort was set up after a series of toxicity events during 1992–1993. This has permitted rapid development of methods for analysis of phycotoxins, and fostered a close working relationship among scientists, industry, and regulators.

An extensive sampling and analysis program, established under the Marine Biotoxin Monitoring Programme, which tested samples from both commercial and recreational harvest sites from around New Zealand's 5650 km coastline, led to the detection of many toxigenic microalgal species and the identification of several incidences of toxin contamination of shellfish (1–5).

The aquaculture industry in New Zealand tests for the presence of amnesic, diarrhetic, neurotoxic, and paralytic shellfish poisoning (ASP, DSP, NSP, and PSP) toxins in shellfish because all 4 toxin groups have been found at levels above maximum permitted limits (MPL) at some time in the past 5 years. Most other countries test for only 1 or 2 of the toxin groups, most commonly PSP and DSP. Currently, testing involves acidic aqueous (PSP, ASP) or acetone (NSP, DSP) extraction of shellfish samples followed by the mouse bioassay to detect PSP, NSP, or DSP toxin; ASP toxins are detected by liquid chromatography (LC). Processing of the acetone extract for the mouse bioassay is unsuited to large-scale screening as it requires liquid–liquid partitioning into dichloromethane, evaporation to dryness, and reconstitution in Tween 20. Samples with positive mouse bioassays for the acetone extracts are re-extracted with ether and retested by mouse bioassay and DSP enzyme-linked immunosorbent assay (ELISA) to confirm the presence of either NSP or DSP toxins. Retesting also ensures that positive results are not due to the presence of acetone-soluble bioactive compounds such as gymnodimine or free fatty acids which are of low toxicity when administered orally. The multiple extraction and analytical techniques place a very heavy demand on the regulatory laboratories and make this an expensive system, considering that more than 99.5% of samples analyzed in New Zealand are negative or below MPL.

The industry, in conjunction with the New Zealand regulatory authorities, has determined that it would be more efficient and less costly to introduce a screening test to identify samples that are toxin-free, as against those which are toxin-positive. Shellfish growing areas classed as toxin-free could be harvested without further testing, whereas toxin-positive areas would be subjected to the accepted regulatory testing method to determine whether toxin levels exceeded MPL.

A New Zealand industry-wide working group has proposed the following operating parameters for the screening system: (*1*) a single simple extraction procedure; (*2*) minimum sample size consistent with representative sampling; (*3*) extraction and analysis to capture all 4 toxin groups (Table 1); (*4*) assay to give Yes/No answer for toxin presence; (*5*) absence of false negatives; (*6*) assay to require no animals (i.e., no mouse bioassays); (*7*) assay to give fast results (<20 h) with high throughput (35 samples/day or about 100 samples/week); (*8*) applicable to all commercial shellfish. An overriding requirement was that the system

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Table 1. Toxins that need to be detected, and compounds that may interfere in bioassay or are similar in structure, but should not be detected by the screening system

Toxin	Detected	Not detected
DSP	Okadaic acid DTX-1, 2, & 3	Free fatty acids
	Yessotoxins[a]	
	Pectenotoxins[a]	
ASP	Domoic acid	Kainic acid
PSP	All 21 STXs	Ciguatera
NSP	All brevetoxins	Gymnodimine

[a] Under review (34).

be cost effective, resulting in a reduction in the total cost of product testing.

After a review of the literature on toxin extraction and analysis, the following system (Figure 1) was proposed: homogenize shellfish sample (100 g) in 90% ethanol (500 mL) and centrifuge to clarify the supernatant; dilute a sample of the supernatant (e.g., 50-fold) with buffer to avoid matrix interference; analyze with an array of ELISAs (full 96-well plate format, with all standards and samples in duplicate); re-analyze all samples above preset limits with the approved regulatory analytical technique for the toxin detected (e.g., mouse bioassay for PSP and NSP; LC for domoic acid); subject a proportion of negative samples to full regulatory testing to ensure that false negatives are not occurring.

Instrumental analytical techniques such as LC, mass spectrometry, and capillary electrophoresis were excluded as screening methods because they require expensive equipment and do not provide the required daily sample throughput. Of the remaining assays [neuroblastoma sodium channel bioassay (6), cytotoxicity bioassay (7), receptor binding assays (8–10), enzyme (protein phosphatase) inhibition (11, 12), and ELISA (13–20)], that might meet these criteria, only ELISA is applicable to all 4 toxin classes (Table 2). Also, ELISAs are relatively cheap and quick (therefore suited to handling large numbers of samples), do not require sophisticated and expensive facilities, and can be automated. In addition, ELISAs can be further developed for accurate quantitation of toxin concentration, and validated for regulatory analysis.

Although individual alternative techniques might have advantages over the corresponding ELISA, adoption of several

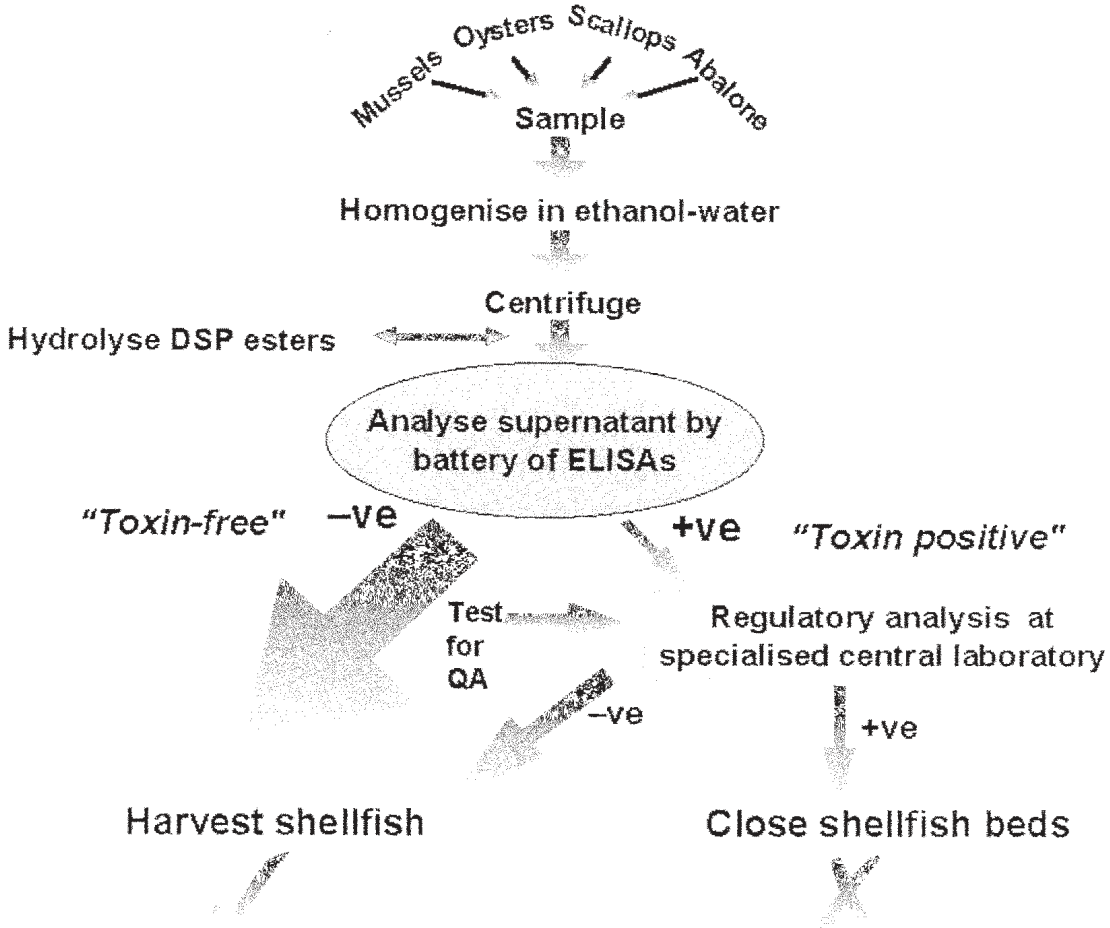

Figure 1. Schematic for proposed screening system.

Table 2. Assays available for detecting algal toxins

Assay	DSP	ASP	PSP	NSP
Neuroblastoma bioassay			✓	✓
Cytotoxicity bioassay	✓			
Receptor binding assay		✓	✓	✓
ELISA	✓	✓	✓	✓
Enzyme inhibition assay	✓			

Table 3. Cross-reactivities (%) of STX and neo-STX antibodies to PSP toxins[a]

Toxin	STX antibody	Neo-STX antibody
STX	100	29
GTX-2/3	11	Detect
dc-STX	29	3.3
Neo-STX	2	100

[a] Data from refs. 15, 36, 37.

different techniques would substantially increase the capital and staff costs of establishing a screening system. The neuroblastoma bioassays for NSP and PSP (6), and the cytotoxicity assay for DSP (7) impose considerable extra costs for the staff training and facilities required to maintain mammalian cell cultures, and receptor binding assays (8–10) require the use of radioisotopes and expensive liquid scintillation counting equipment.

Most of these assay techniques (except ELISA) detect toxic activity (not necessarily caused by the target toxin) rather than the presence of a particular toxin. Although this can be considered an advantage by giving greater assurance of consumer safety, false positives can lead to the unnecessary closure of shellfish beds. In contrast, ELISAs specifically recognize the presence of particular toxins. This specificity is ELISA's greatest disadvantage, as some antibodies fail to recognize all members of a toxin family and thereby underestimate the total toxin content. This is a well-recognized problem with PSP toxins, where saxitoxin (STX) antibodies have poor cross-reactivity with neo-STX and vice versa (Table 3). The specificity of the ELISA must be determined and appropriate antibodies incorporated into the ELISA for each toxin class before an assay is selected for incorporation into the screening system.

Two options are available to circumvent this problem. In the first, a single ELISA is used to identify toxin-contaminated samples for further analysis, using a cut-off concentration set on the assumption that the toxin present is the one least recognized by the antibody. With this option, the assay sensitivity for the toxin must be high enough that if only this toxin is present, the ELISA would still give a positive result. This approach would detect all contaminated samples but would also lead to a relatively high proportion of false positives, because shellfish may retain low levels of better-recognized toxins for some time after ingesting toxic algae [e.g., retention of PSP toxins (21)]. For example, using the STX ELISA alone, 1 ng STX, 9 ng gonyautoxin (GTX)-2/3, and 50 ng neo-STX will all return a value of 1 ng STX equivalents. The second and preferred option is to use 2 or more ELISAs based on antibody recognition of different members of the same toxin family. Chu et al. (22) demonstrated the feasibility of this approach, finding a very high correlation between the results of analyzing PSP-contaminated samples by mouse bioassay and by totaling the results of STX and neo-STX ELISAs. The ease and speed of ELISA analyses makes it relatively simple to conduct several ELISAs in parallel (testing for the 4 toxin families would, in any case, require 4 ELISAs), and the use of 2 ELISAs to cover a particular toxin group requires little extra work. To confirm the feasibility of this scheme, it is necessary to demonstrate that a single solvent extraction system adequately extracts all toxins, and that assays with suitable sensitivity and specificity, free of matrix interference from the shellfish extracts, are available.

The extraction of several of the target toxins by either methanol or ethanol has been reported (18, 23, 24), suggesting that these solvents might be suitable if they gave good recovery of all toxins and did not cause matrix interferences in the ELISA (recovery efficiency was not often reported). Similarly, although ELISAs (14, 15, 19, 20, 22) or commercial ELISA test kits (PSP - Ridascreen, R-Biopharm, Darmstadt, Germany; DSP, SCETI Laboratories LTD, Tokyo, Japan; Rougier Biotech, Montreal, Canada; and Iatron Laboratories, Chiyoda-ku, Tokyo, Japan) were available for all 4 toxin families (Table 4), the assays had been optimized for different extraction procedures and solvents, and not all were available in the preferred 96-well format. It was therefore necessary to demonstrate that the ELISAs were sufficiently sensitive to detect the target toxin in the common extract at concentrations below the regulatory level after dilution, and to develop an ELISA for the DSP toxins in the required 96-well format.

Experimental

Extraction Procedure

Toxin recoveries were determined by spiking shellfish samples (1 ± 0.05 g) with the target toxin before homogenization in 5 mL 90% ethanol–water, followed by centrifugation (10 min at $3000 \times g$). The clarified supernatant was diluted at least 1/50 with phosphate-buffered saline (PBS; 0.01M phosphate, pH 7.4, containing 0.15M NaCl) and analyzed by ELISA. The standard curves were fitted using Microplate Manager software (BioRad, Richmond, CA) and the unknown concentrations were determined by the same program. ELISA standard curves were prepared in ethanol–PBS (1 + 9) or an appropriately diluted extract of toxin-free shellfish of the same species unless specified otherwise.

Table 4. ELISA available for use in the shellfish screening program

Toxin	Assay developer[a]	Antibody source
Domoic acid	AgResearch (20)	AgResearch (20)
STX	AgResearch	Munich University (14)
	Ridascreen kit	R-Biopharm, Germany
Neo-STX	FS Chu, Wisconsin	Fs Chu, Wisconsin (15)
PbTx-2,3	AgResearch (19)	M. Poli, USAMRIID (38)
	AgResearch	AgResearch (25)
Okadaic acid	DSP-check test kit	SCETI Laboratories, Japan (28)
	Okadaic acid ELISA test kit	Rougier Biotech, Canada
	OA-check test kit	Iatron Labs, Japan (17)
	AgResearch	T. Uda, Japan (28)
YTX	AgResearch	AgResearch (26)

[a] Reference numbers in parentheses.

Assay Development and Optimization

Assays for the various toxin classes were developed and applied by using the following reagents and procedures; emphasis was placed on keeping the methodology and all reagents as similar as possible in the assay procedures.

Domoic acid was detected by ELISA using the method of Garthwaite et al. (20). PSP toxins were detected by an STX ELISA using antibody and reagents provided by G. Terplan, Ludwig Maximilians University, Munich, Germany (14). Brevetoxin ELISA initially used antibody and reagents donated by M. Poli, USAMRIID, Fort Detrick, Frederick, MD, in an assay developed in our laboratory (19). This, however, incorporated an amplification step which increased both the cost and time required for each analysis. Therefore, a direct competitive ELISA was established with antibodies raised in sheep (25). An indirect competitive okadaic acid ELISA was established using antibodies raised by Shestowsky et al. (16) obtained from Accurate Chemical & Scientific Corp., Westbury, NY, and an OVA–okadaic acid conjugate developed in-house. To increase the sensitivity for dinophysis toxin (DTX) analogues, the antibody reagent was replaced by antibodies supplied by SCETI Laboratories. A yessotoxin (YTX) ELISA was established with reagents developed in-house (26). Each ELISA was optimized for maximum sensitivity using standard procedures: for the indirect ELISA, checkerboard titrations of antibody and plate-coating conjugate were performed. These were followed by titration of second antibody and competition studies using free toxin to determine the reagent preparation giving greatest sensitivity, accuracy, assay robustness, and precision; direct ELISAs were optimized by titrating antibody coating concentration against increasing concentrations of toxin–enzyme conjugate with, and without, free toxin as competitor (27). Reference toxin solutions were diluted with ethanol–PBS to ensure maximum ethanol concentration in the assay of 10% (v/v) for all assays except the DSP (okadaic acid) ELISA in which alcohol concentrations of 18% were used.

Results and Discussion

The potential of ethanol and methanol as universal extraction solvent was investigated. Ethanol was, and is, the preferred solvent because of its lower toxicity and volatility. In most of the assays, it caused fewer matrix effects than methanol; however, methanol was used to investigate DSP. A decision on the final solvent of choice for the screening system has yet to be made. Toxin recoveries >90% were obtained for domoic acid (20), and preliminary studies showed good recovery of brevetoxin (PbTx-2), indicating that the proposed extraction is suitable for use in the screening system for both aqueous and lipophilic toxins.

Matrix effects of alcohol extracts in the ELISA were eliminated by diluting the shellfish extract with PBS until coincidence of the standard curves was obtained for toxins dissolved in PBS–ethanol (9 + 1) and in PBS-diluted shellfish extract. This procedure was repeated for each of the major commercial shellfish species (green lipped mussel, *Perna canaliculus*; oyster, *Tiostrea chilensis, Crassostrea gigas*; scallop, *Pecten novaezealandiae*) for the domoic acid ELISA, and is in progress for the remaining ELISAs. The domoic acid ELISA requires dilution of the ethanolic extract 1/50 with PBS, and early indications are that the brevetoxin ELISA requires a dilution of extract of 1/100 for removal of matrix effects. Results to date indicate that matrix effects can be avoided in the DSP assay by diluting methanol extracts 1/5, if compared with standards prepared in 18% aqueous methanol (26). Matrix effects are removed from the YTX assay by diluting the extract 1/250.

The minimum dilution required to overcome matrix effects, in conjunction with the MPL for each toxin, effectively determines the assay sensitivity required. The ELISA must be capable of detecting the toxin at or below the MPL in the shell-

Table 5. Comparison of required assay sensitivity with working range of available ELISAs

	Maximum permitted limit (MPL)			ELISA working range	Suitability for screening system
Toxin	MPL μg/100 g flesh	Dilution needed for assay	ng/mL diluted extract[a]	ng/mL extract	Detection below MPL
ASP (domoic acid)	2000	1/50	80	0.02–100	✓
PSP (STX)	80[b]	(1/250)[c]	0.64	0.005–0.075	✓
Neo-STX	" "	(1/250)[c]	0.64	0.5–30[d]	✓
NSP (PbTx-2, 3)	80[e]	1/100	1.6	0.5–20	✓
DSP (okadaic acid)	16[f]	1/5	6.4	0.5–13	✓
YTX	100[g]	1/250	0.8	0.03–1	✓

[a] Calculated for stated dilution of original shellfish sample from standard extract (100 g homogenized with 500 mL ethanol).
[b] As STX equivalents.
[c] Calculated for worst case scenario of 1/250; assay sensitivity is easily able to cope with this.
[d] Data from Chu et al. (22).
[e] MPL assuming 1 MU = 4 μg PbTx-2 (personal communication from T. Yasumoto).
[f] MPL (Europe; personal communication from M.L. Frenandez, EU Reference Laboratory, Vigo, Spain).
[g] Recommended interim MPL from roundtable discussion at VIII International Conference on harmful algae, Vigo, Spain (39). Currently included in DSP toxin regulation: "A tolerable level of DSP toxins, including nondiarrhetic acetone-soluble toxins of 20–40 MU/kg of whole shellfish meat." The no-observable effect level (NOEL) of YTX administered orally to mice is >1 mg/kg; LD_{50} mouse i.p. 100 μg/kg (34).

fish after the extract is diluted to remove matrix effects. The MPL for each toxin group, the equivalent toxin concentration in the diluted extract, and the working range for each ELISA are listed in Table 5.

Assays of suitable sensitivity and cross-reactivity are now available, or potentially available (there is limited resource of neo-STX antibody), for ASP, NSP, and PSP toxins (Table 5), but each will require the common extract to be individually diluted to match toxin concentration with assay sensitivity, i.e., to mitigate the effect of matrix and to bring the extract concentration within the assay working range. An alternative to high dilution would be the removal of interfering compounds by incorporating a simple solvent partition. To improve the detection limit for NSP toxins, we are currently investigating a partition step which uses a highly nonpolar solvent, e.g., hexane to remove interfering fatty acids.

For the DSP group of toxins, the ELISA must detect okadaic acid and the DTX toxins. Ideally, the ELISA would detect these toxins equally. Such an ELISA, however, is unavailable. We used an ELISA based on antibodies with high cross-reactivities to DTX-1 and DTX-2 (28). Until a broader specificity ELISA becomes available, detection of DTX-3 requires the introduction of a base hydrolysis step into the extract preparation to remove the acyl group and convert the toxin to DTX-1. This allows the assay of DTX-3 with sufficient sensitivity by the current ELISA. The simple base hydrolysis procedure of Suzuki et al. (29) was incorporated into our extraction protocol for screening DTX toxins. NaOH (2.5M) is added to the methanol extract in the ratio 1:8, and the reaction is allowed to stand at 78°C for 40 min. The extract is neutralized and diluted for analysis.

Commercial DSP test kits, which have limits of quantitation for okadaic acid ranging from 9 to 20 ng/mL, are available from SCETI Laboratories, Rougier Biotech, and Iatron Laboratories. The kits, developed in conjunction with extraction systems optimized for use with the okadaic acid ELISA in isolation, have been successfully used for screening for okadiac acid (OA) and DTX toxins mainly in shellfish hepatopancreas (30–33). They have limited sensitivity when applied to whole shellfish testing, which is customary in New Zealand, and the goal of the screening system.

Conclusions

The results of this study indicate that the establishment of an ELISA-based screening system for identifying suspect shellfish samples is entirely feasible. We demonstrated that the common extraction protocol is able to extract all toxin groups, and that the extract can be processed through a battery of ELISAs with sufficient sensitivity to detect toxin levels below the MPL of each toxin group.

The incorporation of a base hydrolysis step resolves the problem of detecting DTX- analogues in the short term; a longer term goal would incorporate ELISAs with broader specificity for toxins of this group. A YTX ELISA may be incorporated into the screen to facilitate separation of this bioactive from the DSP group, where it may have been falsely placed due to its activity in the mouse bioassay (34). To avoid the potential problem of high false-negative or false-positive rates that might arise if PSP toxin detection relies solely on an STX ELISA with poor sensitivity for neo-STX, the extracts should be analyzed by using a neo-STX ELISA and STX ELISA in

parallel. The ease of performing ELISAs allows for ready expansion of the system to screen for other toxins and bioactive compounds that may come under regulatory requirements, once ELISAs for them become available, e.g., pectenotoxin, ciguatera (35). Pectenotoxin ELISA is now also established by AgResearch (40). For a review of PSP ELISA, *see* Usleber et al. (41).

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