SPECIAL GUEST EDITOR SECTION

Immunobiosensor Detection of Domoic Acid as a Screening Test in Bivalve Molluscs: Comparison with Liquid Chromatography-Based Analysis

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A rapid and sensitive immuno-based screening method was developed to detect domoic acid (DA) present in extracts of shellfish species using a surface plasmon resonance-based optical biosensor. A rabbit polyclonal antibody raised against DA was mixed with standard or sample extracts and allowed to interact with DA immobilized onto a sensor chip surface. The characterization of the antibody strongly suggested high cross-reactivity with DA and important isomers of the toxin. The binding of this antibody to the sensor chip surface was inhibited in the presence of DA in either standard solutions or sample extracts. The DA chip surface proved to be highly stable, achieving approximately 800 analyses per chip without any loss of surface activity. A single analytical cycle (sample injection, chip regeneration, and system wash) took 10 min to complete. Sample analysis (scallops, mussels, cockles, oysters) was achieved by simple extraction with methanol. These extracts were then filtered and diluted before analysis. Detection limits in the ng/g range were achieved by the assay; however, the assay parameters chosen allowed the test to be performed most accurately at the European Union's official action limit for DA of 20 μg/g. At this concentration, intra- and interassay variations were measured for a range of shellfish species and ranged from 4.5 to 7.4% and 2.3 to 9.7%, respectively.

Domoic acid (DA) is a neurotoxin responsible for incidents of shellfish poisoning (1). The first indication of DA intoxication was reported in 1987 in Canada (2). It is a naturally occurring amino acid that acts as an agonist to glutamate, a neurotransmitter in the central nervous system. DA is produced by at least 2 species of red algae (3) that are ingested by the shellfish during normal filter feeding.

As a result of the significant risk to human health posed by DA, monitoring of toxin concentrations in edible shellfish is required by European Directives 91/492/EEC and 2002/226/EC (4). The current action limit is set at 20 μg DA/g of whole body. As DA has a complicated mode of action, bioassays tend to lack the required sensitivity, and liquid chromatography (LC) is the method of choice (5). Although sufficiently sensitive and specific, the LC procedures tend to be cumbersome, and lengthy sample preparation is required before analysis. Both capillary electrophoresis (6) and mass spectrometry (7) have been developed to detect DA but the methods tend to suffer the same disadvantages as LC.

The inherent properties of immunoassays such as enzyme-linked immunosorbent assays (ELISA) appear to offer an attractive alternative to the physiochemical approach of LC analysis. A number of workers have explored the potential development of antibodies to DA and subsequent production of ELISA-based assays (8, 9). These studies have suggested that immuno-based approaches could be suitable for mass screening, although they lack a full method comparison with the standard LC procedure. More recently, the use of immunosensor technology has been investigated as a possible alternative to conventional testing procedures. Kreuzer et al. (10) developed an electrochemical sensor for DA toxin analysis. This technique proved to have high sensitivity but suffered from poor precision.

The present study outlines the development and validation of a fast and simple screening procedure for DA in shellfish using a commercial, optically based surface plasmon resonance (SPR) biosensor system. Because of the small molecular weight of DA, an indirect assay was used, i.e., the toxin was immobilized onto the chip surface. The developed procedure was compared with the results obtained by LC analysis on a range of different types of shellfish samples.

Routine Testing for Domoic Acid

Council Directive 91/492/EEC, as amended by Directive 97/61/EC, lays down the hygiene requirements for the production and marketing of live bivalve molluscs. These directives are implemented in Northern Ireland by the Food

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Safety (Fisheries Products and Live Shellfish/Hygiene) Regulations (NI) 1998 as amended. As part of the controls to protect public health, the Directive requires periodic monitoring of relaying and production areas to check for the presence of toxin-producing plankton in the water and biotoxins in shellfish. Shellfish samples (oyster, cockle, mussel, and scallop) are analyzed for the presence of Diarrhetic Shellfish Toxins, Paralytic Shellfish Poisons, and Amnesic Shellfish Poisons (DA). Whole shellfish samples are analyzed for the presence of biotoxins on a monthly basis. When samples with above-the-specified-threshold levels are detected, sampling frequency for positive sites is increased to weekly, until 2 consecutive negative results are obtained.

Experimental

Apparatus and Reagents

(**a**) *Optical SPR biosensor system (Biacore Q)*.—Equipped with control and evaluation software (Biacore AB, Uppsala, Sweden).

(**b**) *CM5 sensor chips, HBS-EP buffer, and amine coupling kit*.—Obtained from Biacore AB.

(**c**) *Solutions*.—DA; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); *N*-hydroxysuccinimide (NHS); human serum albumin (HSA); Freund's complete adjuvant and Freund's incomplete adjuvant were purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK). All solvents were of LC grade and were obtained from Rathburn (Walkerburn, Strathclyde, UK). MES buffer was 0.05 M 2-(*N*-morpholino) ethanesulfonic acid (MES) and 0.5 M sodium chloride, pH adjusted to 6.

(**d**) *CRM-ASP-MUS-B*.—Certified reference shellfish material was obtained from the National Research Council, Halifax, Canada. Negative shellfish were chosen from our routine sampling procedures after LC confirmatory analysis.

Preparation of Domoic Acid–Human Serum Albumin Immunogen

EDC (3.5 mg) and NHS (2.1 mg) were added to a solution of HSA (10 mg) in 1.0 mL MES buffer and mixed for 15 min at room temperature (RT). DA (3.5 mg) was added to the activated HSA solution. The reaction mixture was incubated overnight at RT before purification by extensive dialysis against saline (0.15 M sodium chloride). The purified immunogen was diluted with saline to a final concentration of 1 mg/mL protein and stored at –20°C until used.

Antibody Production

A polyclonal antibody was raised in a rabbit by subcutaneous injection with 0.2 mg DA immunogen emulsified with Freund's complete adjuvant. Booster injections (0.2 mg immunogen emulsified with Freund's incomplete adjuvant) were administered on a fortnightly basis. Test bleeds were collected 2 weeks after each booster injection and monitored for the presence of antibodies by immunoassay. After 7 booster injections, the antiserum was harvested and stored frozen at –20°C until required.

Immobilization of Domoic Acid to the Surface of a CM5 Sensor Chip

DA was immobilized to the surface of a CM5 sensor chip externally from the instrument. Briefly, the chip surface was activated by contact with 50 μL of a 1:1 mixture of 0.4 M EDC:0.2 M NHS for 20 min. The reactants were removed and 50 μL 1 M ethylenediamine added and allowed to remain in contact with the sensor chip surface for 1 h. The chip was then removed and 50 μL of 1 M ethanolamine solution was added to the surface for 20 min; the surface was then washed with deionized water and dried with a stream of nitrogen gas. EDC (5 mg) and NHS (2 mg) were dissolved in 450 μL 10 mM sodium acetate pH 4.5, and mixed with 2 mg DA dissolved in 550 μL water. An aliquot (50 μL) of this mixture was added to the chip surface and allowed to react for 2 h. The reactants were then removed from the chip surface; the surface was washed with deionized water and dried, and the sensor chip was stored desiccated at +4°C.

Sample Preparation

A 1 g portion of known negative tissue homogenate (i.e., from shellfish tested negative for DA by LC) was weighed into glass bottles and subsequently spiked with 50 μL of the appropriate standard concentrations (10, 20, 50, 100 μg/50 μL) of DA to create a calibration curve. These fortified samples were treated identically to all test samples. Methanol (10 mL) was added to all bottles and mixed on a Vortex mixer for 5 s before roller-mixing for 30 min. After centrifugation at 3500 × *g* for 10 min at 10°C, an aliquot of each supernatant (1 mL) was transferred into glass tubes and evaporated to dryness at 80°C under a gentle stream of nitrogen. The residue was reconstituted in 1 mL HBS–EP, mixed on a Vortex mixer for 15 s, transferred to Eppendorf tubes, and centrifuged for 5 min before passage through a 0.22 μm filter. The extract was then diluted 1:40 in HBS–EP buffer before analysis.

Analysis

The diluted shellfish extract was mixed 1:9 (v/v) with DA antibody diluted 1:400 in HBS–EP buffer. Each sample (25 μL) was injected over the sensor chip surface at a flow rate of 25 μL/min. Report points were recorded before and after each injection. The chip surface was regenerated with a 25 μL injection (flow rate, 25 μL/min) of 100 mM sodium hydroxide.

Validation

The specificity of the selected antibody was tested by spiking blank mussel samples with saxitoxin diacetate, kainic acid, and L-aspartic acid. The calibration curves obtained with these were compared to those produced with DA-spiked samples.

Mussel, oyster, and cockle samples ($n = 20$) determined to be free of DA by LC were assayed by the biosensor procedure to determine their respective limits of detection (LOD). The various types of shellfish samples were reanalyzed after

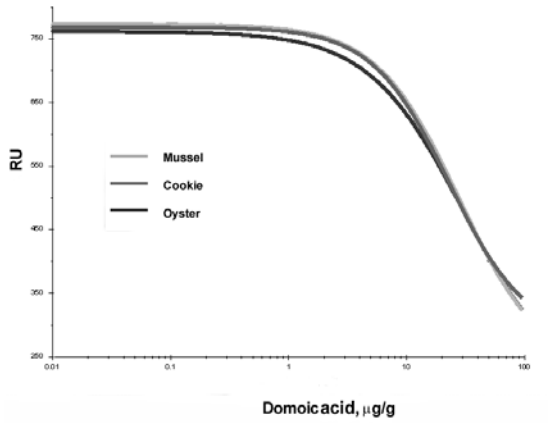

Figure 1. Biosensor calibration curves for DA obtained after extraction from mussel, oyster, and cockle tissue.

spiking with 20 μg/g DA to determine the detection capability for each shellfish species.

Intra- (n = 6) and interassay variations (n = 3) were assessed for mussel, oyster, and cockle tissue replicates spiked with DA at 0, 10, 20, and 40 μg/g. Interassay variation (n = 8) was assessed for 2 different incurred scallop samples that had previously been evaluated by the LC method to contain DA concentrations of 16.84 and 47.98 μg/g, respectively. For the purpose of method comparison (biosensor vs LC), the concentrations of DA present in 78 scallop samples and 20 samples of cockle, mussel, and oyster samples were analyzed by both methods. All samples were stored frozen (–20°) until analysis was completed.

A certified reference material containing DA (CRM-ASP-MUS-B) was used as part of the validation process to determine the accuracy of the DA concentrations measured by the biosensor procedure. CRM-ASP-MUS-B is a mussel tissue (*Mytilus edulis* L.) containing certified levels of DA (36 ± 1 μg/g) and its diastereoisomer epidomoic acid (3.0 ± 0.4 μg/g) as well as several other isomers, giving an overall total of 44.2 ± 1 μg/g DA.

The independent LC method used for comparison consisted of a methanol–water sample extraction, strong anion exchange cleanup, and isocratic analysis, as described by Lawrence et al. (5).

Results and Discussion

Assay Validation

The polyclonal antiserum raised was found to be highly specific to DA. No significant cross-reactivity was found with any of the other compounds included in the assessment.

The average concentration (n = 12) of DA detected by the biosensor analysis of the CRM-ASP-MUS-B after extraction was 47.4 ± 3.7 μg/g with a relative standard deviation (RSD) of 7.8%. The DA concentration of the CRM-ASP-MUS-B was determined by biosensor analysis to within 7% of the certified DA concentration (44.2 μg/g). This result strongly suggests that the antibody used in the present study has significant cross-reactivity with the major isomers of DA present in the CRM and that the biosensor assay developed provides an accurate measure of total DA present in samples.

During biosensor assay development studies, it was found that alternative parameters to those described previously (e.g., antibody dilution and flow rate) could achieve a very high degree of sensitivity [inhibiting concentration (IC_{50}) of 0.07 μg/g]. Further improvements in sensitivity could still be achieved by further parameter manipulation. However, because the official reporting action limit for DA has been set at 20 μg/g, it was concluded that having the midpoint of the calibration curve close to the action limit would produce an assay with the optimum performance with regard to accuracy at the action limit. Calibration curves (Figure 1) were constructed in DA-free mussel, oyster, and cockle samples using the assay conditions described. Using these conditions, IC_{50}s of 18.9, 18.3, and 17.9 μg/g were obtained for mussel, oyster, and cockle tissues, respectively.

Locally harvested scallop material was found to be unsuitable for this calibration study, as many batches were found to have low concentrations of DA present (as measured by LC).

Each functionalized flowcell on the surface of the biosensor chip remained stable for up to 200 sample injections. Four separate flowcells were available on the CM5 chips used in this present study, allowing up to 800 analyses to be performed per chip.

Analysis of 20 known negative mussels, oysters, and cockles gave LOD (mean + 3 SD) values as 1.06, 4.88, and 7.02 μg/g, respectively, when analyzed against a mussel matrix curve. The LODs for oysters and cockles were further calculated as 3.53 and 0.48 μg/g, respectively, when assayed against their equivalent shellfish matrix curve. From these results, it was shown that cockle extracts have a more

Table 1. Intra-assay parameters calculated for DA biosensor assay (n = 6)

		Spiking concentration DA, μg/g			
Shellfish		0[a]	10	20	40
Mussels	Mean, μg/g	1.46	12.9	21.09	37.43
	SD	0.85	0.61	1.56	5.57
	RSD, %	—[b]	4.7	7.4	14.9
Cockles	Mean, μg/g	0.38	14.07	26.45	46.68
	SD	0.31	0.81	1.19	2.71
	RSD, %	—	5.8	4.5	5.8
Oysters	Mean, μg/g	4.18	8.26	16.92	40.09
	SD	2.31	1.04	1.02	3.24
	RSD, %	—	12.6	6.0	8.1

[a] Values obtained with 0 spiking concentrations relate to the background matrix effect for each type of sample applied to the assay.

[b] — = Nonapplicable.

Table 2. Interassay parameters calculated for domoic acid biosensor assay (n = 3)

Shellfish		Spiking concentration DA, μg/g			
		0[a]	10	20	40
Mussels	Mean, μg/g	1.24	10.64	18.44	35.77
	SD	0.46	1.23	1.40	6.05
	RSD, %	—[b]	11.6	7.6	16.9
Cockles	Mean, μg/g	0.67	11.68	20.88	38.19
	SD	0.62	0.75	2.03	7.27
	RSD, %	—	6.4	9.7	19.0
Oysters	Mean, μg/g	1.25	9.96	18.60	34.69
	SD	1.11	0.13	0.43	6.09
	RSD, %	—	1.3	2.3	17.6

[a] Values obtained with 0 spiking concentrations relate to the background matrix effect for each type of sample applied to the assay.

[b] — = Nonapplicable.

pronounced matrix effect than either mussel or oyster extracts. However, all LOD values obtained using the mussel matrix were well below the 20 μg/g threshold established in legislation (4). It was therefore concluded that this procedure, i.e., using mussel extracts to prepare calibrants, was suitable for the analysis of DA in mussel, oyster, and cockle samples.

Twenty negative samples of mussel, oyster, and cockle were fortified at 20 μg/g and assayed in the biosensor assay. Mean concentrations of 21.1, 21.2, and 19.5 μg/g and RSD values of 15.7, 12.9, and 8.7% were obtained in the 3 matrixes, respectively. No significant differences were seen in these positive results when assayed against calibrants prepared in their own shellfish type.

All fortified samples were declared positive (i.e., above their relevant LODs), allowing the detection capability (CCβ) of the assay to be set at 20 μg/g. The intra- and interassay data calculated for each shellfish type at the relevant concentrations are outlined in Tables 1 and 2, respectively. At the DA action limit of 20 μg/g, RSDs ranged from 4.5 to 7.4% and 2.3 to 9.7%, respectively. The interassay data calculated for the incurred scallop samples gave mean concentrations of 66.0 and 18.8 μg/g with RSD values of 9.6 and 13.7%, respectively.

Method Comparison

King scallops harvested from the coastal waters of Northern Ireland over a 10-month period (n = 78) were analyzed by both LC and biosensor procedures (Figure 2). The data generated by the 2 analytical procedures showed a good degree of correlation (r^2 = 0.94). In 65% of all scallop samples analyzed, the biosensor assay yielded higher DA concentrations than the LC procedure. The most likely reason for this finding is that the biosensor assay detects DA, epidomoic acid, and the other DA isomers, whereas the routine LC method determines only the presence of DA and epidomoic acid. Further evidence of this theory was found during the CRM-ASP-MUS-B results reported previously. This apparent slight overestimation by the biosensor procedure was considered advantageous, as the possibility of the method generating a false-negative result was greatly diminished.

The purpose of the present study was to develop a reliable screening procedure for DA toxin in locally harvested shellfish. One of the most important characteristics of a screening test is the avoidance of false-negative results. To minimize the possibility of false-negative results with the biosensor assay, it was decided to reduce the action limit from 20 to 15 μg DA/g of whole flesh. Any samples found to contain >15 μg DA/g whole flesh were subjected to confirmatory analysis by the LC method.

Of all scallop samples analyzed in the present study, 69% contained DA levels above the biosensor action limit and 95% of those were consequently determined by LC to be above the regulatory action limit of 20 μg/g. Twenty samples of mussel, oyster, and cockles were also randomly chosen over the same 10-month sampling period and tested by both methods. DA concentrations above the action limits of either method were not found in any of these samples. Thus, no false-negative results were observed for the biosensor assay during this study. In the case of scallop samples, a 5% false-positive rate was assigned to the sensor screening procedure (based on LC comparative data).

The comparative study of the 2 methods (biosensor and LC) was taken beyond the issues regarding analytical performance. The speed of analysis, another key performance indicator of a screening method, was compared between the 2 procedures. Results of this investigation are shown in Table 3. The main findings were that the sample batch size by biosensor analysis was twice that achievable by LC. An additional time saving was found in the time it took to make the Biacore Q sensor ready for analysis. Thus, for this instrument, the change from a particular analyte chip to another takes a few minutes, whereas LC set-up procedures

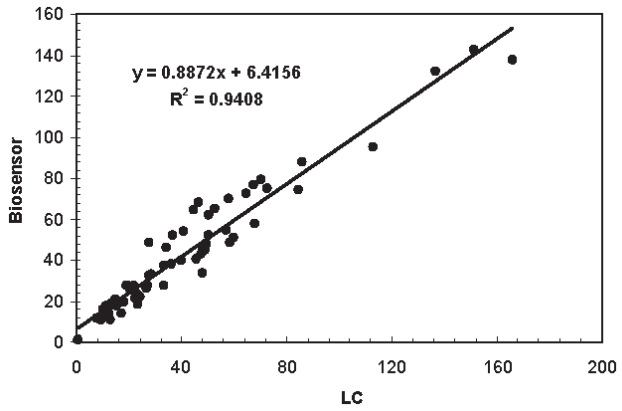

Figure 2. Results obtained from analysis of 78 scallop samples by biosensor and LC procedures (μg/g).

Table 3. Comparison of biosensor and LC analysis times for a maximum batch of shellfish samples

	Biacore Q	LC
Maximum sample numbers per batch	20	10
Preparation of extracts, h	4	5.5
Switchover time[a], h	0.1	1.5
Analysis time, h	4	3
Result calculation, h	Nil	1
Time per sample, h	0.4	1.1
Total time[b], h	8.1	11

[a] Time taken to prepare instrument for DA sample analysis.
[b] Time taken from beginning of sample extraction to result.

are more manipulative. This biosensor "chip in, chip out" capability has been shown to greatly increase the versatility of the technique to allow several different forms of analysis to be performed within a single day.

The development, validation, and comparison of the biosensor assay with an LC procedure has had a 2-fold impact on the ability to screen shellfish for DA presence. The main function of a screening test is to remove negative samples from further investigation and to allow positive samples to be confirmed by more time-consuming and expensive techniques. The large majority of samples analyzed routinely as part of the marine toxin monitoring program are determined to contain undetectable levels of DA, or concentrations below the defined action limit. The implementation of this rapid biosensor method as a screening test considerably reduced the number of samples requiring solid-phase extraction and subsequent analysis by LC. The biosensor method has reduced shellfish analysis time by about 60% per sample when compared to LC analysis. This in turn has facilitated a larger throughput of samples and reduced the length of time the product must be held in storage, allowing prompt reporting of official DA results and issue of the related documentation demanded by the monitoring bodies.

The biosensor assay described has been shown to be suitable for use as a screening test with a zero rate of false negatives to detect DA at the action level for whole body in shellfish. Up to 20 shellfish homogenates could be extracted in a single working day. Such innovative technologies should be considered a welcome addition to routine testing laboratories where analyses must be completed within strict time limits.

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