Enzyme Immunoassay for the Determination of Domoic Acid in Mussel Extracts[†]

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A competitive enzyme immunoassay (ELISA) was developed for measuring the concentration of domoic acid in extracts of mussel tissue. The assay employed a polyclonal serum raised in mice against an ovalbumin–domoic acid conjugate. Spike–recovery experiments demonstrated that the concentration of domoic acid in both aqueous and acid extracts of mussel tissue could be accurately measured to within 8% of the actual value with the ELISA. The ELISA yielded accurate determinations at concentrations as low as 0.25 µg of domoic acid/mL of extract. Results of HPLC and ELISA analyses of sea mussel extracts correlated well ($r = 0.96$), although the ELISA resulted in higher values for domoic acid concentration than the HPLC for most samples. This was partially attributed to a loss of domoic acid in the solid phase extraction prior to HPLC and to the possible presence of domoic acid isomers. It was concluded that a procedure such as the ELISA described herein would provide a useful complement to the standard HPLC procedure currently employed in routine domoic acid analyses.

Keywords: *Domoic acid; immunoassay; ELISA; shellfish toxins; HPLC*

INTRODUCTION

Domoic acid, a neuroexcitatory toxin produced by the marine diatom *Pseudonitzshia pungens*, can contaminate edible shellfish and is the causal agent of amnesic shellfish poisoning (ASP). Like paralytic shellfish poisoning (PSP), ASP poses serious public health concerns. In December 1987, a domoic acid outbreak in cultured mussels on Prince Edward Island resulted in 107 human cases of ASP, including four deaths and several cases of permanent memory loss (Todd, 1989). The 1987 ASP outbreak had a devastating impact on the entire East Coast shellfish industry and nearly precipitated its collapse.

Both *P. pungens* and domoic acid-contaminated shellfish have since been found in the waters off the West Coast of North America. Furthermore, shellfish harvest closures in both eastern and western Canadian fisheries have heightened public awareness of the problem in recent years. The seafood industry worldwide now views domoic acid and the potential for ASP outbreaks as a serious economic threat. As a consequence, the demand for domoic acid testing is rapidly growing.

In Canada, the quality of shellfish for human consumption is assured in part by the Marine Toxin Monitoring Program, administered by the Inspection Branch of the Department of Fisheries and Oceans. Shellfish extracts are routinely analyzed for domoic acid using standard high-performance liquid chromatographic (HPLC) methods. While highly accurate and reproducible, HPLC methods can be expensive and time-consuming. Sensitive and cost-effective methods complementary to HPLC methods could be useful in processing the sheer volume of samples requiring domoic acid analysis. Immunochemical methods, while generally meeting these criteria, can be adapted to formats which

facilitate the rapid, simultaneous screening of many samples. Moreover, they have the further advantage of portability and, unlike HPLC procedures, can be modified for use in field testing.

This paper describes a competitive enzyme-linked immunosorbent assay (ELISA) for determining domoic acid concentrations in mussel extracts. The major objectives of this study were, first, to evaluate the accuracy of this ELISA by using it to determine the concentration of domoic acid in mussel extracts pre-spiked with known levels and, second, to compare the ELISA with the standard HPLC method in determining domoic acid concentrations in extracts prepared from naturally contaminated mussels.

MATERIALS AND METHODS

Mussel Samples. Mussels for the spike–recovery experiments were harvested on Prince Edward Island and purchased from a local market. Sea mussels used to compare domoic acid determinations by the ELISA and standard HPLC methods were harvested between September 26 and December 26, 1993, from the Barkley Sound region (management area 23) of Vancouver Island, BC. MUS-1, a mussel homogenate contaminated with well-characterized levels of domoic acid and domoic acid isomers (Wright *et al.*, 1989, 1990; Quilliam, 1991), was provided by the Marine Analytical Chemistry Standards Program of the Institute for Marine Biosciences, National Research Council of Canada, Halifax, NS.

Extraction and Sample Preparation. A bottle of MUS-1 mussel homogenate (15.17 g) was processed according to the aqueous extraction method of Quilliam *et al.* (1989a). Briefly, the mussel homogenate was mixed 1:1 with distilled water and boiled for 5 min. This was centrifuged (10000*g*, 15 min). The supernatant was collected and the pellet washed with an additional 15 mL. The final volume of the extract was made up to 50.0 mL. The total domoic acid concentration in the MUS-1 extract was 39.1 + 1.8 µg/mL, as indicated by the MUS-1 certification update material (Quilliam, 1991). The MUS-1 extract was analyzed for domoic acid content using the competitive ELISA.

Purchased mussels were shucked, and 74 g of meat was homogenized in a blender with two 30 s bursts. One 15 g portion was removed and an aqueous extract prepared (Quil-

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liam et al., 1989a). A second 15 g portion of mussel homogenate was extracted according to the AOAC (1990) acid extraction procedure, in which 0.18 N HCl was mixed 1:1 with the mussel homogenate and boiled for 5 min. The supernatant, referred to as the extract, was collected by centrifugation.

Aliquots of both the aqueous and acid mussel extracts were spiked with the extracted MUS-1 material to final total domoic acid concentrations of 0.25, 0.5, 1.0, 5.0, and 10.0 µg/mL and analyzed with the competitive ELISA. Extraction of the sea mussel samples was performed by the Inspection and Special Services Branch of Fisheries and Oceans Canada, Burnaby, BC. Samples 19885, 19884, 19881, and 20053 were extracted according to the AOAC (1990) procedure. Mussel tissues from all other samples were homogenized and extracted in one part water and two parts methanol (Quilliam et al., 1991). Solids were removed by centrifugation and analyzed by HPLC and ELISA.

HPLC. HPLC analyses of sea mussel extracts for domoic acid were performed at the Inspection and Special Services Branch of Fisheries and Oceans Canada (Burnaby, BC), after the method of Quilliam et al. (1991). Briefly, a 2 mL aliquot of each extract was slowly run through an LC-SAX solid phase extraction (SPE) tube (Supelco, Oakville, ON) preconditioned with 50% methanol and water. The tubes were rinsed with 5 mL of 10% acetonitrile followed by 0.5 mL of 12.2% triammonium citrate in 10% acetonitrile (citrate buffer). Citrate buffer was continuously poured through the SPE tube until 2.0 mL of extract was collected in a volumetric tube. This was transferred to an autosampler vial and analyzed for domoic acid by HPLC using a Novapak C_{18} column (3.9 × 150 mm). The mobile phases consisted of 0.02% phosphoric acid (A) and acetonitrile (B); the concentration of A was decreased in a linear fashion from 95% to 65%, while that of B was increased from 5% to 35% over 15 min. Eluate was monitored using diode array detection at 239 nm. DACS-1 (domoic acid calibration solution; NRC) was used as a standard. HPLC analyses were performed without prior knowledge of the ELISA results.

Competitive ELISA. *Domoic Acid Conjugation.* Domoic acid was conjugated to keyhole limpet hemocyanin (KLH) and ovalbumin (OVA) using a carbodiimide reaction as previously described (Smith and Kitts, 1994). Both protein carriers, the carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), required buffers and desalting columns were purchased from the Pierce Chemical Co. (Rockford, IL). Briefly, 2 mg of KLH and 2 mg of OVA were each dissolved in 200 µL of deionized water. Domoic acid (1 mg; Sigma Chemical Co., St. Louis, MO) was dissolved in 520 µL of conjugation buffer (Pierce) and was added to each of the KLH and OVA solutions. To the vial containing KLH was added 50 µL of an EDC stock solution (10 mg/mL). The contents of the vial containing OVA, however, were added directly to a second vial containing 10 mg of EDC. Both preparations were incubated for 2 h at room temperature. Precipitates were removed from both conjugates by centrifugation, and free domoic acid was removed with a Presto desalting column (Pierce) equilibrated with conjugation buffer. Protein-containing fractions of both conjugates were concentrated with Centricon 30 ultrafiltration devices (Amicon, Beverly, MA). To ensure complete removal of free domoic acid, both retentates were again desalted. Protein-containing fractions were pooled, filter sterilized, aliquotted into sterile vials, and stored at 4 °C. Protein concentrations of both conjugates were determined according to the method of Bradford (1976).

Antisera Production. Male Balb/C mice were given one injection of the OVA–domoic acid conjugate (approximately 100 µg) mixed 1:1 with Freund's incomplete adjuvant (Sigma). This was followed by four intraperitoneal injections of 30 µg of OVA–domoic acid conjugate containing no adjuvant, spaced 10–14 days apart. Four days prior to sacrifice, a final intraperitoneal injection of 80 µg of OVA–domoic acid conjugate was delivered, again with no adjuvant. Throughout the immunization period, the mice displayed neither signs of distress nor any of the typical symptoms of ASP. The serum, heretofore referred to as anti-OVA–DA, was collected from the heart following euthanasia.

Table 1. Spike–Recovery Experiment of Domoic Acid in Aqueous and Acid Sea Mussel Extracts As Determined by Competitive ELISA

domoic acid spiked (µg/mL)	domoic acid determined[a] (µg/mL; % recovery in parentheses) in	
	aqueous extract	acid extract
39.1[b]	41.0 ± 1.60 (105)	
10	9.75 ± 0.82 (97.5)	10.8 ± 0.84 (108)
5	4.95 ± 0.24 (99.0)	4.59 ± 0.39 (91.8)
1.0	1.08 ± 0.30 (108)	1.06 ± 0.22 (106)
0.5	0.53 ± 0.06 (106)	0.50 ± 0.04 (100)
0.25	0.26 ± 0.08 (104)	0.25 ± 0.00 (100)

[a] Mean and standard deviation of triplicate measurements over at least two dilutions. [b] MUS-1 standard extract.

ELISA Procedure. Falcon (Becton-Dickson, Lincoln Park, NJ) immunoassay (IA) plates were coated with 100 µL of 0.5 µg/mL KLH–domoic acid conjugate in 100 mM NaHCO₃, pH 9.6, and incubated overnight at 4 °C. Plates were thoroughly washed with UB, which consisted of 10 mM Tris-HCl, pH 8.0 (Bio-Rad Laboratories, Mississauga ON), 250 mM NaCl (Fisher Scientific, Vancouver, BC), 1 mM MgCl₂ (BDH, Vancouver, BC), 0.05% NaN₃ (Fisher), and Tween 20 (Bio-Rad), after each step. The IA plates were blocked with 200 µL/well 5% skim milk powder (Carnation, Toronto, ON) UB (without the Tween 20) and incubated for 1 h at 37 °C.

Anti-OVA–DA was prepared by diluting the crude serum 1/2000 into a solution of UB with 1% skim milk powder. Standards and sample extracts were diluted into this solution in preparation for domoic acid analysis. The domoic acid standard consisted of the DACS-1 at final concentrations ranging between 1 and 200 ng/mL and measured in triplicate. Aqueous sample extracts (Quilliam et al., 1989a) were diluted between 1/25 and 1/500. Acid sample extracts (AOAC, 1990) were diluted at least 1/50. ELISA determinations were performed without prior knowledge of the HPLC determinations. Domoic acid concentrations in all samples were determined in triplicate at each of two or three dilutions. A preparation of a nonrelevant mouse serum was used in blank wells to determine background absorbance. Sample and standard preparations were added to the plates and incubated for 2 h at 37 °C. Goat anti-mouse IgG alkaline phosphatase conjugate (GAMIG-AP; Bio/Can, Mississauga, ON) was diluted 1/3000 in UB with 1% skim milk powder and added to the plates (100 µL/well). Following a 1 h incubation at 37 °C a 0.5 mg/mL solution of p-nitrophenyl phosphate (Sigma) was added (100 µL/well) and incubated at 37 °C. Absorbances were measured after 1 h at 405 nm with an interference wavelength of 655 nm using a Model 450 microplate reader (Bio-Rad) and corrected for background absorbance. Sample domoic acid concentrations were determined from a standard curve relating standard domoic acid concentration and absorbance, generated on every plate.

RESULTS AND DISCUSSION

Experiments were performed to test the competitive ELISA in determining accurately the concentration of domoic acid in an extract of MUS-1 and both aqueous and acid extracts of uncontaminated mussel tissue. These extracts were spiked with various proportions of MUS-1, giving final concentrations of 0.25–10 µg of domoic acid/mL of extract.

The concentration of domoic acid in MUS-1 was accurately determined to within 5% of the actual value in the aqueous extract (Table 1) with DACS-1 used as a standard. The domoic acid contents of DACS-1 and MUS-1 have been extensively analyzed and characterized (Quilliam et al., 1989a,b; Pleasance et al., 1990; Wright et al., 1989, 1990). Notably, the isomeric profiles of the DACS-1 standard and the MUS-1 differ considerably. DACS-1 was found to consist of 98% domoic acid (Figure 1a), with the diastereoisomer (Figure 1b) com-
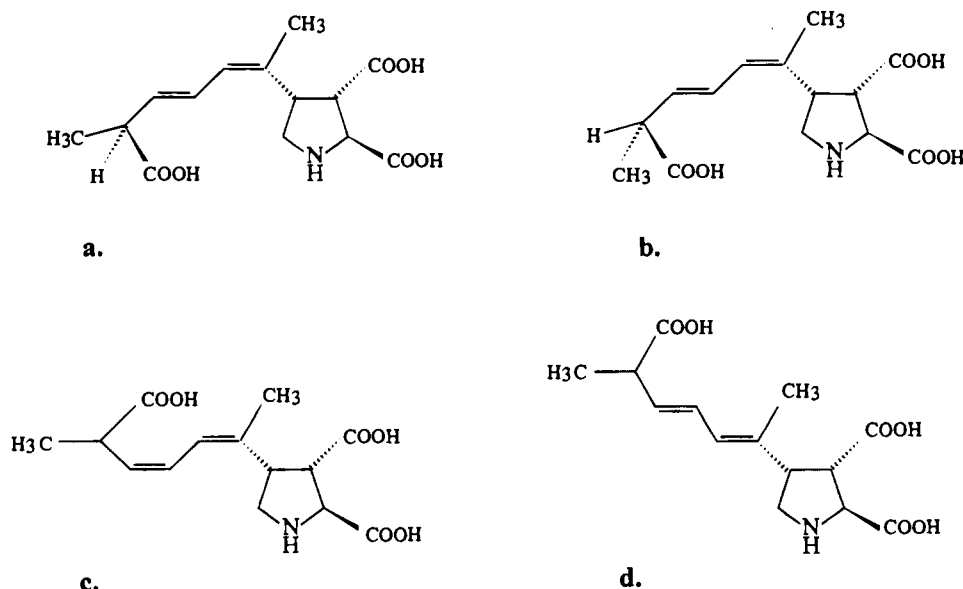
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Figure 1. Chemical structures of (a) domoic acid, (b) the diastereoisomer of domoic acid, and (c and d) *cis−trans* isomers of domoic acid.

prising the remaining 2%. The total domoic acid content of MUS-1, however, was found to consist of 76% domoic acid (Figure 1a), with 12% comprised of the diastereoisomer of domoic acid (Figure 1b), 7.8% (Figure 1c) comprised of a *cis−trans* isomer, and another *cis−trans* isomer (Figure 1d) comprising an additional 4% (Quilliam, 1991). Because the total domoic acid concentration in MUS-1 was accurately determined using the DACS-1, it would appear that the antiserum cross-reacts well with the major isomers of domoic acid and that this competitive ELISA provides a measure of total domoic acid, which includes the concentration of the various domoic acid isomers (Figure 1).

Total domoic acid concentration was accurately determined to within 8% of the actual value in both aqueous and acid extracts and at 10, 5, 1.0, and 0.5 μg of domoic acid/mL of extract (Table 1). Levels as low as 0.25 μg of domoic acid/mL of mussel extract were accurately determined within 4% of the actual values. This represents 0.5 μg of domoic acid/g of mussel tissue when acid (AOAC) extracts are analyzed. The lower limit of detection for domoic acid in AOAC (1990) acid mussel extracts with HPLC is reported to be 0.5 μg of domoic acid/g of tissue (Lawrence *et al.*, 1989). In the competitive ELISA, the lower limit of detection of domoic acid in mussel extracts was not precisely determined; however, it must be below the accurately determined level of 0.5 μg of domoic acid/g. In sea mussel extracts there are several samples that contained no detectable domoic acid in HPLC, while domoic acid was detected by ELISA (Table 2). At this time it is difficult to assess whether these values are accurate or if they represent levels of domoic acid below the limit of accurate determination or falsely positive reactions due to interfering compounds in the extracts.

Direct comparison of domoic acid determinations with ELISA and HPLC analyses of sea mussel extracts indicated a good correlation ($r = 0.96$) between the two methods (Table 2). In most cases, the ELISA determination yielded higher domoic acid concentrations than HPLC analysis (Table 2). This result may be due to inconsistent recoveries from batch operations using solid phase extraction in preparation for HPLC or to the presence of domoic acid isomers.

Table 2. Comparison of Domoic Acid Determinations Using HPLC and ELISA

	domoic acid[a] (μg/g)	
sample	HPLC	ELISA
20106	undetected	undetected
19761	undetected	0.16 + 0.00
19885	undetected	0.16 + 0.02
19785	undetected	0.24 + 0.04
20093	undetected	0.30 + 0.04
20062	undetected	0.36 + 0.04
20090	undetected	0.36 + 0.12
20092	undetected	0.36 + 0.08
20105	undetected	0.36 + 0.08
19845	undetected	0.36 + 0.08
20164	undetected	0.51 + 0.02
20163	undetected	0.58 + 0.08
19884	undetected	2.80 + 0.28
20107	0.4	1.00 + 0.20
20096	0.7	2.80 + 0.44
19940	0.9	1.40 + 0.20
19844	1.3	2.00 + 0.20
20050	1.4	3.00 + 0.48
20051	1.4	2.00 + 0.40
19981	1.9	2.60 + 0.10
20064	2.1	1.76 + 0.16
20065	2.1	1.56 + 0.04
20095	2.2	2.64 + 0.16
20068	2.2	4.76 + 0.60
20048	3.5	3.80 + 0.30
20067	4.2	5.00 + 0.96
20054	5.2	8.20 + 0.28
19939	7.9	8.70 + 0.84
20030	9.9	12.2 + 0.48
20053	18.2	13.7 + 0.71
19980	47.4	35.4 + 1.80
20029	54.3	70.4 + 1.20

[a] Mean and standard deviation of duplicate or triplicate measurements.

Domoic acid isomers are known to occur in toxic mussels (Quilliam *et al.*, 1989a; Wright *et al.*, 1990). Isomers that do not coelute with domoic acid are not determined in routine HPLC analyses and may have contributed to the apparent discrepancy. The ELISA determination likely represented a measure of the total domoic acid content, including the diastereoisomer (Figure 1b) and at least two *cis−trans* isomers (Figure 1c,d). It should be noted, however, that the actual isomeric contents in these samples were never deter-

mined by HPLC; therefore, this explanation remains unconfirmed.

Finally, although the anti-OVA serum does not cross-react with kainic acid (Smith and Kitts, 1994), the presence of other uncharacterized cross-reacting analogues in some mussel samples remains a possibility, albeit a remote one. Saxitoxin also does not cross-react with the anti-OVA−DA serum (Smith and Kitts, 1994); therefore, it is highly unlikely that paralytic shellfish toxins interfered with the domoic acid ELISA.

The domoic acid content in 2 (no. 19980 and 20053) of the 32 samples was less when determined by ELISA than that determined by HPLC (Table 2). Because domoic acid is known to transform in acidic conditions, particularly when stored above refrigeration temperatures (Lawrence et al., 1989), it is possible that the concentration of domoic acid may have decreased in some samples between the time of the HPLC and ELISA analyses. Underestimation of domoic acid concentration in these mussel extracts could also be due to the presence of compounds that interfere with the HPLC analysis of shellfish extracts for domoic acid. An example of an endogenous compound that coelutes with domoic acid in rancid samples is believed to be a derivative of tryptophan (Quilliam et al., 1989a). The presence of such a compound in the mussel extracts used in this study could also have resulted in an overestimation of domoic acid in HPLC analysis in those particular samples.

The potential presence of the amnesic shellfish toxin in shellfish and crab tissues delineates the necessity for reliable and effective methods of domoic acid detection and quantification to provide an assessment of human risk. The standard procedure used in routine domoic acid determinations is the HPLC method (Quilliam et al., 1991). Of the thousands of samples that are routinely analyzed by HPLC every year as part of marine toxin monitoring programs, the vast majority are determined to contain undetectable levels of domoic acid (Chiang and Loy, 1992, 1993). Although the HPLC method is effective and reliable, the required extraction and cleanup procedure is relatively time-consuming compared to the ELISA procedure in which the extracts are simply diluted into the antibody solution. Moreover, the use of a solid phase extraction step can introduce recovery errors. HPLC and more sophisticated methods such as ion-spray mass spectrometry (Quilliam et al., 1989b) may be used more efficiently in confirming domoic acid contamination in samples that have been prescreened.

Screening of mussel extracts for domoic acid contamination would considerably reduce the number of samples requiring methanol extraction, solid phase extraction, and analysis by HPLC. This would allow a higher turnover of samples testing positive and requiring rapid confirmation by HPLC as well as an increased capacity to test suspect samples of other species. Furthermore, more rapid analyses of commercial shellfish and crab samples would reduce the length of time the product must be held in storage and allow the prompt issue of the official documentation of domoic acid content, which is demanded by some export markets.

There are presently a wide variety of methods available for domoic acid analysis. Each method, because of its individual advantages and disadvantages, is perhaps best suited to particular applications. The AOAC mouse bioassay (AOAC, 1990), modified for ASP detection, relies on specific, behavioral symptoms including scratching and convulsions (Wright et al., 1989) as well as death times. The mouse bioassay for domoic acid has an LD_{50} exceeding 70 µg/mouse and a lower limit of detection of about 40 µg/mouse (Grimmelt et al., 1990). In addition to the obvious ethical problems raised in using animals as indicators of toxicity, this assay is too insensitive for screening shellfish samples for domoic acid contamination, as the legal tolerance limit for domoic acid is 20 µg/g of shellfish tissue.

Radioreceptor assays are highly sensitive and specific for receptor binding compounds and are well suited for use in toxicokinetic studies. A radioreceptor assay for domoic acid, utilizing the competitive binding of [^{3}H]-kainic acid to the kainate glutamate receptor, has recently been reported (Van Dolah et al., 1994). Although this assay is rapid, sensitive, and designed to process many samples simultaneously, its reliance on the use of radioisotopes restricts its use to the research laboratory.

The ELISA for domoic acid described herein, with a lower limit of detection below 0.5 µg/g, would be adequately sensitive for screening large numbers of samples, while the use of radioisotopes is avoided. ELISA methods are also easily adapted to rapid, semi-automated formats, thus facilitating a high throughput of samples. Moreover, such a procedure would be convenient, as AOAC extracts routinely prepared for PSP testing could be directly screened for domoic acid without the need for further processing or cleanup. Domoic acid levels determined by ELISA to be above a particular threshold could be confirmed by HPLC. Although there may be a chance of obtaining false positives at levels below 0.5 µg of domoic acid/g, it is unlikely that this threshold would be set so close to the detection limit of HPLC. ELISA analyses of 32 mussel samples did not fail to detect domoic acid when shown to be present by HPLC analyses; therefore, the occurrence of false negatives in the ELISA for domoic acid appears to be unlikely.

Finally, the flexibility and portability of immunoassays make them ideal for use as on-site testing tools. Given the sporadic nature of domoic acid outbreaks, an on-site field test for this toxin would help to provide more timely analyses, especially in remote areas. Consequently, the monetary losses incurred by harvesters and processors when commercial lots are recalled due to domoic acid contamination could be reduced or avoided.

In summary, the immunochemical method for determining domoic acid concentration in shellfish extracts described herein could provide a rapid on-site field test for domoic acid contamination in shellfish. In its present form it represents a sensitive, accurate, and economical screening method that could effectively complement the standard HPLC procedure currently employed in the routine monitoring for domoic acid in shellfish.

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