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Preparation and Characterization of Domoic Acid–Protein Conjugates Using Small Amount of Toxin in a Reversed Micellar Medium: Application in a Competitive Enzyme-Linked Immunosorbent Assay

Philippe Branaa,[†] Jérôme Naar,[†] Mireille Chinain,[†] and Serge Pauillac*[,†,‡]

Unité d'Océanographie Médicale, Institut de Recherches Médicales Louis Malardé, PO Box 30, Papeete, Tahiti, French Polynesia, Unité d'Immunocytochimie, CNRS URA 359, Département d'Immunologie, Institut Pasteur, Paris, France. Received April 12, 1999; Revised Manuscript Received August 4, 1999

With the aim of producing novel antibodies to domoic acid (DA), an original, rapid, and simple procedure for preparing minute amount of hapten–protein conjugates was developed. The amide-bond-generating mixed anhydride method of Erlanger was performed using 0.32–0.64 μmol of DA in a reversed micellar medium allowing strong carrier haptenization as determined by spectrophotometric measurement. Bovine serum albumin (BSA) and ovalbumin (OVA) conjugates were, respectively, used for immunization of BALB/c mice and antibody screening by enzyme-linked immunosorbent assay (ELISA). Specific polyclonal antibodies were produced upon multiple injections of $(DA)_{17}$–BSA conjugate administered by three different routes: (i) intraperitoneal (i.p.), (ii) intraperitoneal + subcutaneous (i.p. + s.c.), (iii) footpad (f.p.). The i.p. route induced antisera of higher titer (1:350000) than did the other protocols (approximately 1:72900) and was selected throughout further experiments. Using a competitive ELISA format with a peroxidase immunoconjugate and a chromogenic substrate, no significant cross-reactivity was observed with glutamic acid, aspartic acid and kainic acid (KA), a structural analogue of DA. The sensitivity of this assay could be enhanced by 1 order of magnitude by using a β-galactosidase immunoconjugate with a fluorogenic substrate while preserving DA specificity. The calculated dissociation constant (K_D) for the interaction of the antibodies with free DA was 5×10^{-7} M (chromogenic assay) and 5×10^{-8} M (fluorogenic assay). Using the optimized assay the limit of detection (LOD) and the limit of quantitation (LOQ) in the ELISA buffer were 1.4 and 3 ng/mL, respectively. Moreover this assay was found applicable for measuring DA levels in spiked mussel extracts precleaned through a solid-phase extraction column, as a very good correlation ($r^2 = 0.96$) was observed between the actual amounts of DA added and amounts detected by ELISA. Thus, accurate determinations of DA in clean extracts could be achieved between 2 and 180 ng/mL in spiked samples which corresponds to 0.02–1.8 μg/g of original mussel tissue. Owing to the regulation limits of 20 μg DA/g of shellfish tissue, these extraction and assay procedures should provide a useful complement to the standard HPLC analytical technique currently employed in monitoring DA in shellfish tissue.

INTRODUCTION

Domoic acid (DA),[1] a naturally occurring amino acid produced by the marine diatom *Pseudonitzschia pungens*, is the most potent compound of a group of structurally related neurotoxins collectively referred to as kainoids after the name of the original member of the group, kainic acid (KA), isolated from the red macroalga *Digenea simplex* (Murakami et al., 1953). Since the first outbreak of food poisoning that occurred in Canada in 1987, DA has been found to accumulate in edible shellfish (Wright et al., 1989; Iverson et al., 1989; Quilliam and Wright, 1989), causing upon ingestion a human syndrome called Amnesic Shellfish Poisoning (ASP). The clinical picture is characterized by a gastrointestinal distress, followed by neurological symptoms such as headache, confusion, disorientation, and severe deficits in short-term memory (Teitelbaum et al., 1990; Perl et al., 1990). At the molecular level, kainoids have been found to exert

* To whom correspondence should be addressed. Phone: +689 41 64 69. Fax: +689 43 15 90. E-mail: spauillac@malarde.pf.
† Unité d'Océanographie Médicale.
‡ Unité d'Immunocytochimie.

[1]Abbreviations: AOT, aerosol OT or sodium bis(2-ethylhexyl) sulfosuccinate; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DA, domoic acid; DMSO, dimethylsulfoxyde; ELISA, enzyme-linked immunosorbent assay; f.p., footpad; GAM-βGZ, goat anti-mouse IgG heavy and light chain-β-galactosidase conjugate; GAM-PO, goat anti-mouse IgG heavy and light chain-horseradish peroxidase conjugate; IFA, incomplete Freund's adjuvant; i.p., intraperitoneal; i.p. + s.c., intraperitoneal + subcutaneous; KA, kainic acid; KLH, keyhole limpet hemocyanin; LOD, limit of detection; LOQ, limit of quantitation; 4-MUG, 4-methyl umbelliferyl β-D-galactoside; OPD, *o*-phenylenediamine; OVA, egg albumin; PBS, phosphate-buffered saline; PBS-M, 5% skim milk powder in PBS; PBS-T, PBS containing 0.1% Tween 20; PBS-TMB, PBS containing 0.1% Tween 20 plus 1% skim milk powder and 1% BSA; PL, poly-DL-lysine (average mol wt 77 000); TLC, thin-layer chromatography on silica gel.

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membrane depolarizing and neurodegenerative properties via the glutamatergic kainate receptor (Debonnel et al., 1989; Stewart et al., 1990).

Due to public health requirements and seafood industries interests, DA detection has been developed using the mouse bioassay (Iverson et al., 1989) and more specific assays based upon either instrumental method (gas or liquid chromatography coupled or not to mass spectrometry and capillary electrophoresis), or pharmacological and immunochemical techniques [see, for review, Wright and Quilliam (1995)]. Human toxicological data collected from the 1987 ASP Canadian incident have led to the establishment of generally admitted DA regulatory limits of 20 μg/g shellfish tissue. These safe limits, lower than the sensitivity of the mouse bioassay (Grimmelt et al., 1990), together with ethical and practical issues, contribute to exclude its routine use. Indeed routine monitoring of shellfish extracts requires high throughput sample screening with great specificity and sensitivity combined with simplicity, all requirements that only meet with automated binding assays or enzyme-linked immunosorbent assays (ELISA). However, in their present form, the disadvantage of receptor-binding assays is the use of radioactive tracers which imposes the purchase of expensive radiolabeled compounds and necessitates the disposal of radioactive waste. Although specific polyclonal antibodies have been produced (Newsome et al., 1991; Smith and Kitts, 1994, 1995; Osada et al., 1995; Garthwaite et al., 1998), until now there is no fully validated screening immunoassay commercially available for shellfish naturally contaminated with DA. Therefore, immunochemical techniques are to be most actively pursued in order to design a laboratory antibody-based assay that could be scaled up to fit the requirement of a routine cost-effective mass screening of shellfish prior to consumption.

This work describes the production of novel antibodies to DA using fully characterized DA−carrier conjugates obtained by a rapid and simple procedure we have recently designed (Pauillac et al., 1998). This paper is the first to document the production of antibodies to DA using very tiny amount of haptenic material (100−200 μg) in a reversed micellar medium. A preliminary application of these antibodies to competitive ELISA provides the basis to the development of a rapid, simple, specific, and sensitive assay for DA contaminated shellfish.

MATERIALS AND METHODS

Preparation of Conjugates. All chemical reagents, unless otherwise stated, were purchased from Sigma Chemicals Co. (St. Louis, MO). High-quality grade solvents obtained from Prolabo (France) were dried according to standard procedures.

Conjugates were prepared according to a modified version of the mixed anhydride coupling method of Erlanger et al. (1957) as recently described by Pauillac et al. (1998). Briefly, 0.321 or 0.642 μmol of DA were dissolved in 20 μL of dry dimethylsulfoxyde (DMSO), and then a 10-fold molar excess of tributylamine and isobutyl chlorocarbonate was added. The formation of the mixed anhydride was allowed to proceed for 30 min at room temperature. The reaction was monitored by thin-layer chromatography (TLC) run on silica gel 60 F_{254}-precoated plastic foils from Merck (Darmstadt, Germany) using CH_2Cl_2-MeOH (95:5) as developing solvent. UV illumination of the chromatogram showed conversion of DA, which remains at the origin ($R_f \approx 0$), to the mixed anhydride ($R_f = 0.77$) to an extent greater than 95%. The conjugation of activated DA to carriers was carried out in the system of reversed micelles of aerosol OT [AOT or sodium bis(2-ethylhexyl) sulfosuccinate] in heptane (Kabanov et al. 1989; Yatsimirskaya et al., 1993) with relevant modifications for miniaturization purpose and high haptenization yield (Pauillac et al., 1998). Three carriers were used throughout: bovine serum albumin (BSA), ovalbumin (OVA), and poly-DL-lysine (PL, average mol wt 77 000) providing an initial hapten/protein molar ratio around 100.

Control experiments run in parallel, consisting in carrier alone and carrier mixed with non-activated DA, were incubated in the reversed micellar medium under identical conditions.

All carriers in the vials (assay and control) were recovered by acetone precipitation. The precipitates were finally resuspended in 1 mL of distilled water, filter sterilized (0.22 μm), dispensed into sterile tubes, then freeze-dried overnight. Tubes were generally stored at −20 °C until use but some of them were kept at room temperature for subsequent analysis.

Conjugates Analysis. For analytical purpose, carriers from control and assay were resuspended in 1 mL of PBS. Carrier haptenization was assessed spectrophotometrically through a 1 cm light path quartz cuvette with a dual beam spectrophotometer (UV 160A, Shimadzu, Japan). Absorption spectra of all samples were recorded between 200 and 300 nm using a 1:3 (BSA and OVA conjugates) or 1:2 (PL conjugates) dilution in PBS. Concentrations of hapten (read at $\lambda_{max} = 242$ nm) and protein carriers (read at $\lambda_{max} = 280$ nm) were determined according to either standard curves with linear regression in the range 0−20 μg/mL of DA or previously reported extinction coefficients (DA, $\epsilon = 83.83$ mg^{-1} cm^2; BSA, $\epsilon = 0.66$ mg^{-1} cm^2; OVA, $\epsilon = 0.735$ mg^{-1} cm^2). After molar conversion, the degree of hapten conjugation was expressed by the hapten/carrier ratio of the samples, i.e., epitope density of the conjugates. For these calculations, total PL content was considered assuming complete recovery of the conjugate.

Mice Immunization. Three groups of two female BALB/c mice (6−8 weeks of age) were immunized by three different routes: intraperitoneal (i.p.), intraperitoneal + subcutaneous (i.p. + s.c.), and footpad (f.p.). All six animals received a total of six injections of 50 μg $(DA)_{17}$−BSA conjugate emulsified in 100 μL of PBS/adjuvant mixture (1:1). The first injection was made in Freund's complete adjuvant (FCA), and the following in Freund's incomplete adjuvant (FIA) at 3−4 week intervals. Mice were sequentially bled from the retroorbital venous system and pooled sera from each group were tested for anti-DA antibody activity.

Enzyme Immunoassays. The following buffers were used throughout ELISA experiments: phosphate-buffered saline (PBS, 0.01 M KH_2PO_4/K_2HPO_4−150 mM NaCl, pH 7.2) for coating, 5% skim milk powder in PBS as blocking buffer (PBS-M), PBS containing 0.1% Tween 20 (PBS-T) for washing steps, and this latter buffer supplemented with 1% skim milk powder and 1% BSA (PBS-TMB) for dilution of reagents (sera, immunochemicals, or DA standards). All immunochemical reagents, unless otherwise stated, were purchased from Boehringer Mannheim (Indianapolis, IN).

Flat-bottomed polystyrene 96-well Maxisorp Immunoplates (Nunc, Roskilde, Denmark) were coated with 100 μL of $(DA)_{10}$−OVA conjugate (20 ng/mL−1 μg/mL) in PBS by incubation for 1 h at 37 °C then overnight at 4 °C. They were then washed five times with PBS, and the

remaining active sites were blocked by addition of 250 μL of PBS-M. All subsequent incubation steps, unless otherwise stated, were carried out 1 h at 37 °C in precoated plates, which were thoroughly washed with PBS-T after each step.

Kinetics Experiments. For kinetics experiments, pooled sera collected at the indicated time from each 2-mice immunization group were diluted 1:2500 and applied to the plates at 100 μL/well. Bound antibodies were revealed by incubation with 100 μL of goat anti-mouse IgG peroxidase conjugate (GAM-PO) diluted 1:3000 followed by the addition of 100 μL of *o*-phenylenediamine (OPD) as a commercial substrate solution (FAST OPD, Sigma). After 30 min at 37 °C, the enzymatic reaction was terminated by adding 50 μL of 2 M H_2SO_4 and the absorbance at 492 nm was measured using a Multiskan Plus Titerteck (Flow Laboratories, Puteaux, France).

Titration Experiments. For titration experiments, serially diluted pooled immune sera collected after the last boost or control sera (1:300−1:1968300 dilutions) were applied to the plates at 100 μL/well. Bound antibodies were revealed by the successive incubations with GAM-PO and OPD, and the enzymatic reaction terminated as described above after 10 min at 37 °C.

Competitive Experiments. Competitive experiments were conducted using pooled sera collected one week after the last boost from mice immunized according to the i.p. protocol. Pooled sera (1:350000 final dilution) were first preincubated overnight at +4 °C with an equal volume of various concentrations of inhibitors (DA, KA, glutamic acid, and aspartic acid). Samples of these mixtures were transferred (100 μL/well) onto DA-OVA-precoated plates (250 ng/mL). After incubation and washing steps, the antibodies associated with the plates were revealed as described above. In another set of experiments, coating concentration was reduced to 20 ng/mL and bound antibodies were revealed using a fluorescence-based amplification signal by the successive addition of goat anti-mouse IgG−β-galactosidase conjugate (GAM−βGZ; 1:4000 dilution) and 4-methyl umbelliferyl β-D-galactoside (4-MUG) at saturation in 0.1 M phosphate buffer, pH 7.2. Following a 30 min incubation, fluorogenic substrate change was measured using a Dynatech Microfluor Reader with excitation at 365 nm and emission at 450 nm. Recorded values are expressed as relative fluorescence units.

Using both amplification systems, the signals obtained in the presence of various inhibitor concentrations and without inhibitor (maximal signal) are referred to as B and B_0, respectively. The limit of detection (LOD) of each format assay was therefore calculated assuming a decrease of 3 standard deviations from the mean value of B_0 is significant ($n = 12$). Standard curves were generated by plotting the percentage of maximal signal ($\%\ B/B_0$) against the log of free DA concentrations, using the four parameter logistic fitting model provided by the MultiCalc package program (EG & G−Wallac). The limit of quantitation (LOQ) was defined as the DA concentration necessary to produce a 80% decrease in B_0 (IC_{80}).

Finally, the dissociation constant (K_D) of mice antibodies binding to DA was estimated by calculating the slope of the regression line obtained using the simplified mathematical equation of Klotz (1953) proposed by Friguet et al. (1985).

Measurement of DA Levels in Rapid Mussel Extracts. Rapid extraction of blue mussels (*Mytilus edulis*) was performed according to the method developed by Quilliam et al. (1998). Briefly 4 g of uncontaminated mussels was mixed with 16 mL of MeOH−H_2O (1:1) and homogenized thoroughly using a Brinkman Polytron. Particulate material was eliminated by centrifugation at 5000*g* for 10 min and the supernatant filtered through a dry methanol-compatible 0.5 μm filter unit. Five milliliter samples (1 g tissue equivalent) of this clear supernatant were either used without additional treatment (crude extract) or precleaned by passing it through a strong anion-exchange solid-phase extraction column (Supelclean LC−SAX, Supelco Inc., Bellefonte, PA). Following several washing steps, putative DA was eluted from the column using MeOH/H_2O/HCOOH (75:23:2). These precleaned extracts were evaporated to dryness under nitrogen atmosphere then reconstituted in 5 mL of MeCN/H_2O (1:9). Finally, 1 mL of this suspension (0.2 g tissue equivalent) and 1 mL of crude extract (0.2 g tissue equivalent) were spiked with 4 μg of DA analytical standard (DACS-1C, NRC−CNRC, Institute for Marine Biosciences, Halifax, Canada) to generate concentrations ranging from 4000 to 2 ng/mL by serial dilutions in MeCN/H_2O (1:9). The same dilutions of DA analytical standard were directly prepared in MeCN/H_2O (1:9) to serve as calibration solutions. These spiked samples or calibration solutions were mixed with an equal volume of pooled mice immune sera (1:400000 final dilution) and incubated overnight at +4 °C. DA levels were determined in triplicate 50 μL volumes of these mixtures, using the competitive ELISA format and the optimized fluorogenic amplification system as described above.

RESULTS AND DISCUSSION

Preparation and Analysis of the Conjugates. Conjugates were prepared in a reversed micellar medium according to the procedure described in the Materials and Methods. Their recovery and substitution ratio were carefully checked spectrophotometrically by incorporating controls in which carriers have been incubated under identical conditions with nonactivated hapten. DA is a chromophore with a unique absorption peak at 242 nm and a molecular extinction coefficient of 83.83 $mg^{-1}\ cm^2$ at pH 7 (Falk et al., 1989); therefore, proteins (BSA and OVA) but not the polyamino acid (PL) could be also estimated in the conjugates by UV absorbance at 280 nm (aromatic amino acids).

Consistent with former experiments (Pauillac et al., 1998), carrier protein recovery in control and assay tubes was found to be greater than 95%, hence, the whole carrier content (proteins or PL) was taken into account in the calculation of the hapten/carrier molar ratio (i.e., epitope density) of the samples. Another clue is provided by the demonstration that differential absorption spectra of the conjugates and carriers recovered from control experiments were quite similar to those of the free hapten (data not shown). Using a 100:1 hapten to carrier initial molar ratio, the epitope density of OVA, BSA, and PL conjugates was estimated to be around 10, 20, and 30, respectively. For these calculations the assumption was made that the extinction coefficient of DA is not altered by the conjugation process. This technique proceeding in a reversed micellar medium was initially set up for lipophilic carboxylic haptens (Pauillac et al., 1998) but proved to be likewise valuable for hydrophilic haptens (water solubility of DA is 7.6 mg/mL according to Falk et al., 1991). Compared to relatively high hapten quantities (1−12.4 mg of DA) used in previous studies (Newsome et al., 1991; Smith and Kitts, 1994, 1995; Garthwaite et al., 1998), a significant improvement in preparation of well-defined DA−protein conjugates was achieved by our method. Starting from 200 μg of DA, 435 μg of

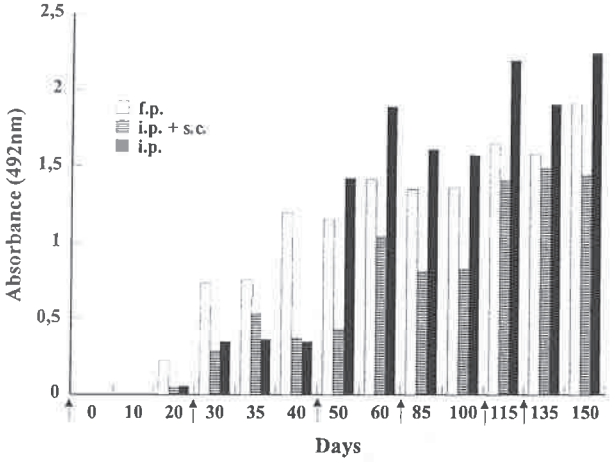

Figure 1. Kinetics of anti-DA antibodies production. Pooled sera from each group were diluted 1:2500 in PBS-TMB and tested for their reactivity onto microtiter plates coated with 1 μg/mL of $(DA)_{10}$–OVA conjugate in PBS. The reaction was developed following the successive incubations with GAM-PO and OPD. Each bar represents the mean of triplicate assays, which varied by no more than 5%. Mice immunization groups: f.p. (open bars), i.p.+ s.c.(hatched bars) and i.p.(black bars). Arrows indicate priming and subsequent boosts.

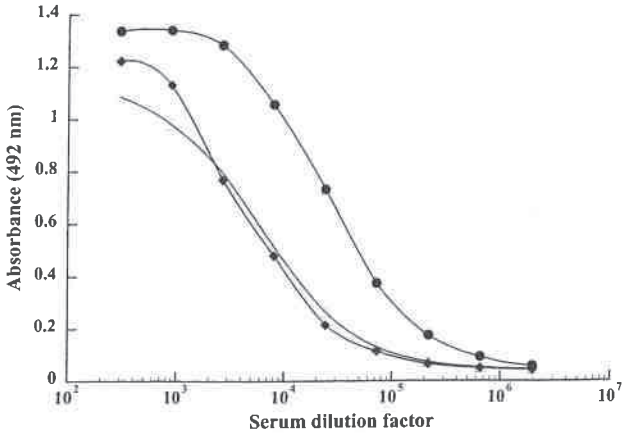

Figure 2. Titration of pooled mice antisera. Pooled sera were serially diluted (1:300–1:1968300) in PBS-TMB and added onto microtiter plates coated with 1 μg/mL of $(DA)_{10}$–OVA conjugate in PBS. The reaction was developed following the successive incubations with GAM-PO and OPD. Mice immunization groups: i.p. (●), i.p.+ s.c. (△), and f.p. (◆). Each value represents the mean of triplicate assays, which varied by no more than 5%.

DA–BSA conjugates were obtained from which 300 μg (six injections of 50 μg) were injected per mouse.

Comparison between the Three Different Routes of Immunization. To investigate the best immunization schedule, three groups of two mice were given a total of six injections of $(DA)_{17}$–BSA conjugates administered via three different routes (i.p., i.p. + s.c., and f.p.). Throughout experiments, mice injected by either route displayed no ill effects or symptoms typical of ASP. Animals were sequentially bled and their sera pooled according to the route of immunization. For each group, anti-DA antibodies production was tested by indirect ELISA using $(DA)_{10}$–OVA conjugate as target antigen. All preimmune sera were totally negative against a panel of coating antigens including the homologous (BSA) and heterologous (OVA) native carriers used (data not shown).

That all mice developed specific immune response upon immunization was clearly demonstrated by the antibody kinetics and level achieved but differences were observed according to the immunization schedule (Figure 1). For instance, the f.p. immunized group responded earlier (as soon as 3 weeks after priming), with a prolonged elevated antibody level attained approximately 3 weeks after the first boost. Consequently subsequent booster injections showed no clear efficacy on further antibody production. In contrast, a secondary immune response of normal magnitude and kinetics could be generated in the i.p. + s.c. and i.p. immunized groups after each injection, especially when the antibody levels had sufficiently decreased before. Nevertheless, the i.p. immunized group always exhibited a stronger specific response, whereas the i.p. + s.c. group tended to catch up with the f.p. one along the time course. These observations were later confirmed by titration experiments (Figure 2). Again, it can be seen that all pooled antisera dilutions exhibited DA specificity, and lower antibody concentrations yielded signals similar to background level (control without antibody). Moreover, it was observed that cross-absorbing anti-DA-BSA antisera with 1% BSA (PBS-TMB diluent buffer) completely neutralized the anti-carrier activity in the sera (data not shown). Therefore, titers estimated by the maximum serum dilution that allowed a 3-fold signal to target antigen, as compared to background level, were found to be 1:350000 for the i.p. mice group and around 1:72900 for the ip. + s.c. and f.p. groups. Consequently, all further reported ELISA experiments were conducted using pooled sera from the last bleeding of i.p. immunized mice.

It is noteworthy that $(DA)_{30}$–PL when used as target antigen yielded a high background noise and nonreproducible results.

Competitive Inhibition of Pooled Mice Antisera Binding to DA-OVA. To further assess the specificity of these anti-DA antibodies, competitive ELISA experiments were performed using various inhibitors (DA, KA, glutamic acid, and aspartic acid). The high specificity of anti-DA antibodies was again clearly demonstrated as all the other free haptens were essentially unrecognized in the concentration range 30–1000 μg/mL (data not shown). Figure 3 illustrates standard curves obtained by plotting the percentage of maximal signal (% B/B_o) against the log of free DA concentrations. These experiments also contributed to compare two amplification signals. The overall sensitivity of the GAM-βGZ/MUG (fluorogenic) amplification system over the GAM-PO/OPD (chromogenic) system is evident from the binding parameters of the standard curves. The working range covered by these assays was 3–245 ng/mL and 58–1437 ng/mL, respectively. Assuming that a difference of 3 standard deviations (n = 12) from the mean signal without inhibitor (B_o) is significant, the LOD of these assays was 1.4 ng/mL and 27 ng/mL using the fluorogenic and the chromogenic amplification systems, respectively. Finally, as can be expected, the K_D for the interaction of the antibodies with free DA was found to be lower using the fluorogenic assay (5×10^{-8} M) than the chromogenic one (5×10^{-7} M).

In the process of the analysis of sera from i.p. immunized mice, two antibody subpopulations were disclosed, which is in good agreement with an earlier observation (Nieto et al., 1984) that decreasing the coating antigen or/and antibody concentrations allowed different antibody subpopulations with increased affinities to be selected.

Recovery of DA from Spiked Samples. The optimized competitive fluorogenic assay has been used to measure DA levels in rapid mussels extracts spiked with DA calibration standards. The effect of matrixes was

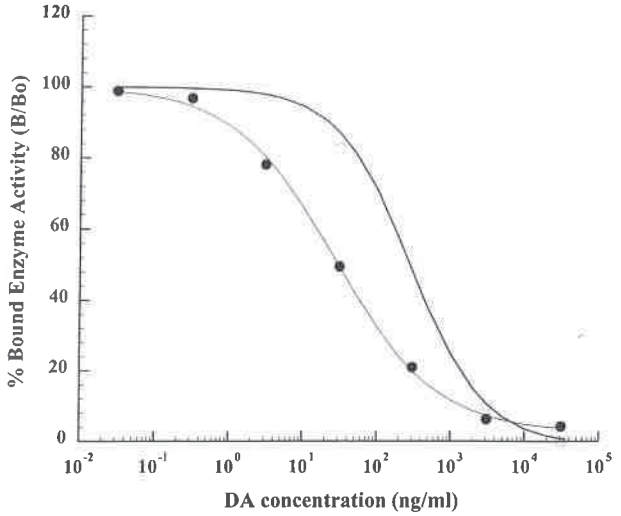

Figure 3. Competitive inhibition of pooled mice antisera binding to DA-OVA coated plates by free DA. $(DA)_{10}$–OVA coating concentration was 250 ng/mL and 20 ng/mL for the chromogenic and fluorogenic assays, respectively. The final antiserum dilution (i.p.-immunized group) was 1:350000 in PBS-TMB. The reaction was developed following the successive incubations of either GAM-PO and OPD (○) or GAM-βGZ and MUG (●) as reported in the Materials and Methods. Each value represents the mean of triplicate assays, which varied by no more than 5%.

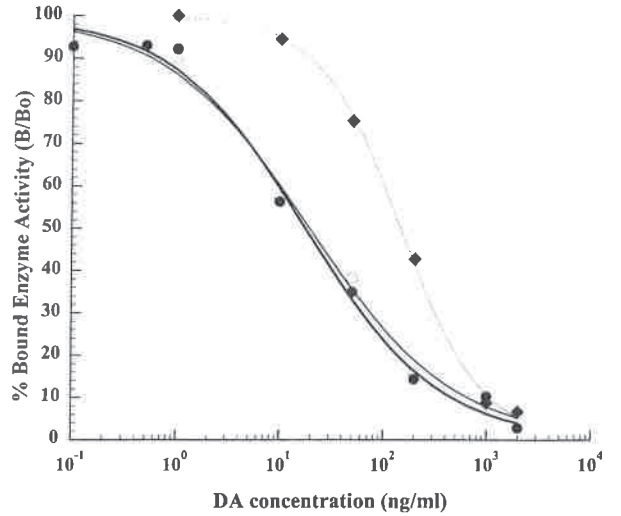

Figure 4. Effect of different biological matrixes on the DA calibration curve using the optimized ELISA format. See legend of Figure 3 for experimental conditions running the fluorogenic amplification system. DA calibration curves prepared in diluent buffer (●), in spiked mussel extracts: crude (◆) and pre-cleaned (○). Each value represents the mean of triplicate assays, which varied by no more than 3%.

investigated by comparing standard calibration curve obtained in diluent buffer with DA recovery from spiked mussel extracts (crude or precleaned). No significant matrix effect was observed for the precleaned extracts ($r^2 = 0.96$), as shown in Figure 4, whereas the use of crude extracts caused negative interferences (i.e., DA is less available for antibody interaction). Consequently, accurate determinations of DA in precleaned extracts could be achieved between 2 and 180 ng/mL in spiked samples, which corresponds to 0.02–1.8 μg/g of original mussel tissue. Owing to the regulation limits of 20 μg of DA/g of shellfish tissue, these extraction and assay procedures should provide a useful complement to the standard HPLC analytical technique currently employed in monitoring DA in shellfish tissue.

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LITERATURE CITED

Debonnel, G., Beauchesne, L., and de Montigny, C. (1989) Domoic acid, the alleged "mussel toxin", might produce its neurotoxic effect through kainic acid receptor activation: an electrophysiological study in the rat dorsal hyppocampus. *Can. J. Physiol. Pharmacol. 67*, 29–33.

Erlanger, B. F., Borek, F., Beiser, S. M., and Lieberman, S. (1957) Steroid-protein conjugates. I. Preparation and characterization of conjugates of bovine serum albumin with testosterone and with cortisone. *J. Biol. Chem. 228*, 713–727.

Falk, M., Walter, J. A., and Wiseman, P. W. (1989) Ultraviolet spectrum of domoic acid. *Can. J. Chem. 67*, 1421–1425.

Falk, M., Seto, P. F., and Walter, J. A. (1991) Solubility of domoic acid in water and nonaqueous solvents. *Can. J. Chem. 69*, 1740–1744.

Friguet, B., Chaffotte, A. F., Djavadi-Ohaniance, L., and Goldberg, M. (1985) Measurement of the true affinity constant in solution of antigen–antibody complexes by enzyme-linked immunosorbent assay. *J. Immunol. Methods 77*, 305–319.

Garthwaite, I., Ross, K. M., Miles, C. O., Hansen, R. P., Foster, D., Wilkins, A. L., and Towers, N. R. (1998) Polyclonal antibodies to domoic acid and their use in immunoasays for domoic acid in seawater and shellfish. *Nat. Toxins 6*, 93–104.

Grimmelt, B., Nijjar, M. S., Brown, J., Macnair, N., Wagner, S., Johnson, G. R., and Amend, J. F. (1990) Relationship between domoic acid levels in the blue mussel (*Mytilus edulis*) and toxicity in mice. *Toxicon* 28, 501–508.

Iverson, F., Truelove, J., Nera, E., Tryphonas, L., Campbell, J., and Lok, E. (1989) Domoic acid poisoning and mussel-associated intoxication: preliminary investigations into the response of mice and rats to toxic mussel extract. *Food Chem. Toxicol. 27*, 377–384.

Kabanov, A. V., Ovcharenko, A. V., Melik-Hubarov, N. S., Bannikov, A. I., Alakhov, V. Yu., Kiselev, V. I., Sveshnikov, P. G., Kiselev, O. I., Levashov, A. V., and Severin, E. S. (1989) Fatty acid acylated antibodies against virus suppress its reproduction in cells. *FEBS 250* (2), 238–240.

Klotz, I. M. (1953) *The Proteins* (H. Neurath and K. Baily, Eds.) Vol. 1, Academic Press, New York.

Murakami, S., Takemoto, T., and Shimizu, Z. (1953) Studies on the effective principles of Digenea simplex Aq. I. Separation of the effective fraction by liquid chromatography. *J. Pharm. Soc. Jpn. 73*, 1026–1028.

Newsome, H., Truelove, J., Hierlihy, L., and Collins, P. (1991) Determination of domoic acid in serum and urine by immunochemical analysis. *Bull. Environ. Contam. Toxicol. 47*, 329–334.

Nieto, A., Gaya, A., Jansa, M., Moreno, C., and Vives, J. (1984) Direct measurement of antibody affinity distribution by hapten-inhibition enzyme immunoassay. *Mol. Immunol. 21* (6), 537–543.

Osada, M., Marks, L. J., and Stewart, J. E. (1995) Determination of domoic acid by two different versions of a competitive enzyme-linked immunosorbent assay (ELISA). *Bull. Environ. Contam. Toxicol. 54*, 797–804.

Pauillac, S, Naar, J., Branaa, P., and Chinain, M. (1998) An improved method for the production of antibodies to lipophilic carboxylic hapten using small amount of hapten-carrier conjugates. *J. Immunol. Methods 220*, 105–114.

Perl, T. M., Bédard, L., Kosatsky, T., Hockin, J. C., Todd, E., and Remis, R. S. (1990) An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *New Engl. J. Med. 322*, 1775–1780.

Quilliam, M. A., and Wright, J. L. C. (1989) The amnesic shellfish poisoning mystery. *Anal. Chem. 61* (18), 1053A–1060A.

Quilliam, M. A., Thomas, K., and Wright, J. L. C. (1998) Analysis of domoic acid in shellfish by thin-layer chromatography. *Nat. Toxins* 6, 147−152.

Smith, D. S., and Kitts, D. D. (1994) A competitive enzyme-linked immunoassay for domoic acid determination in human body fluids. *Food Chem. Toxicol. 32*, 1147−1154.

Smith, D. S., and Kitts, D. D. (1995) Enzyme immunoassay for the determination of domoic acid in mussel extracts. *J. Agric. Food Chem. 43*, 367−371.

Stewart, G. R., Zorumski, C. F., Price, M. T., and Olney, J. W. (1990) Domoic acid: A dementia-inducing exitotoxic food poison with kainic acid receptor specificity. *Exp. Neurol. 110*, 127−138.

Teitelbaum, J. S., Zatorre, R. J., Carpenter, S., Gendron, D., Evans, A. C., Gjedde, A., and Cashman, N. R. (1990) Neurologic sequelae of domoic acid intoxication due to the ingestion of contaminated mussels. *New Engl. J. Med. 322*, 1781−1787.

Wright, J. L. C., and Quilliam M. A. (1995) Methods for domoic acid, the amnesic shellfish poisoning (ASP) toxin. In *Manual on Harmful Marine. IOC Manual and Guides* (G. M. Hallegraeff, D. M. Anderson, and A. D. Cembella, Eds.) vol. 33, pp 115−135, Paris, IOC-UNESCO.

Wright, J. L. C., Boyd, R. K., de Freitas, A. S. W., Falk, M., Foxall, R. A., Jamieson, W. D., Laycock, M. V., McCulloch, A. W., McInnes, A. G., Odense, P., Pathak, V., Quilliam, M. A., Ragan, M. A., Sim, P. G., Thibault, P., Walter, J. A., Gilgan, M., Richard, D. J. A., and Dewar, D. (1989) Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern Prince Edward Island. *Can. J. Chem. 67*, 481−490.

Yatsimirskaya, E. A., Gavrilova, E. M., Egorov, A. M., and Levashov, A. V. (1993) Preparation of conjugates of progesterone with bovine serum albumin in the reversed micellar medium. *Steroids 58* (11), 547−550.

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