Screening of Paralytic Shellfish Posioning Toxins in Naturally Occurring Samples with Three Different Direct Competitive Enzyme-Linked Immunosorbent Assays

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Among three direct competitive enzyme-linked immunosorbent assays (dc-ELISA) tested, a protocol using either anti-saxitoxin (STX)/*STX-horseradish peroxidase (HRP)* or anti-neo-STX/*STX-HRP* pairs was found to be most effective for screening paralytic shellfish poisoning (PSP) toxins in contaminated shellfish. An excellent agreement between the total PSP toxins (STX plus neo-STX levels) obtained by ELISA and by mouse assay was found. Analysis of 1540 naturally contaminated samples revealed that 211 (13.6%) samples were positive by the ELISA as compared with 175 (11.3%) by the mouse assays at the threshold level of 80 μg/100 g of sample. The distributions of the toxins at levels >40 and >80 μg/100 g in 1540 samples obtained by different ELISA formats are presented. The total PSP toxin levels (STX plus neo-STX) obtained from simultaneous analysis of both STX and neo-STX by ELISA are valid for accurate screening for the presence of PSP toxins in these samples and could eliminate as much as 80−85% of the samples needed to run a mouse assay.

Keywords: *ELISA; saxitoxin; neosaxitoxin; PSP*

INTRODUCTION

Paralytical shellfish poisoning (PSP) is one of the most potent naturally occurring food poisonings. The poison constitutes a group of >20 structurally related toxins produced predominantly by the dinoflagellate *Alexandrium (= Protogonyaulax) catenella* and *Alexandrium tamarense*; saxitoxin (STX) and neosaxitoxin (neo-STX) are most toxic and more commonly occur in shellfish such as mussels, clams, and other marine animals (Anderson, 1994; Hall et al., 1990; Schantz, 1979). Both STX and neo-STX have also been found to be produced by the freshwater cyanobacterium (blue green alga) *Aphanizomenon flos-aquae* (Mahmood and Carmichael, 1986). The toxins have little adverse effect on the shellfish, but human ingestion of the toxic shellfish may result in PSP and sometimes can be fatal. Because of the potential health hazard to humans and animals, a quick, sensitive, and specific method is needed to determine the presence of toxins in shellfish. Several methods, including mouse bioassays (Hollingworth and Wekell, 1990), a receptor binding assay (Davio and Fontelo, 1984; Smith and Kitts; 1994; Vieytes et al., 1993), tissue culture assays (Gallacher and Birkbeck, 1992; Jellett et al., 1995; Manger et al., 1993), and chemical methods have been used for the analysis of PSP toxins (Hollingworth and Wekell, 1990; Hungerford and Wekell, 1992; Luckas, 1992). However, most chemical methods are time consuming and require expensive instrumentation (Lawrence and Menard, 1991; Luckas, 1992; Mirocha et al., 1992; Oshima, 1995; Pleasance et al., 1992; Sullivan et al., 1988; Thibault et al., 1991); bioassays, however, are nonspecific and sometimes insensitive. Investigations in our laboratory and by others have led to the development of simple immunoassays for monitoring these toxins in shellfish (Carlson et al., 1984; Cembella et al., 1989; Chu and Fan, 1985; Chu et al., 1992; Hack et al., 1990; Hokama and Smith, 1990; Hout et al., 1989; Johnson et al., 1964; Renz and Terplan, 1988; Usleber et al., 1991, 1994). Commercial enzyme-linked immunosorbent assay (ELISA) kits are also available (for example, Inst. Armand-Frappier, Laval, PQ, Canada, and R-Biopharm, GmbH, Darmstadt, Germany). However, because the specificity of the antibodies cannot cover a wide range of different toxins, problems in using ELISA for PSP toxins still exist. In a recent study, we found that two separate direct competitive (dc) ELISAs, one using antibodies specific for STX and one using those specific for neo-STX, were necessary to detect the overall PSP levels present in the shellfish (Huang et al., 1996). In the present study, a simplified approach that involved two specific antibodies (anti-STX and anti-neo-STX) but one toxin-enzyme marker (STX-enzyme conjugate) in the ELISA was developed. A total of 1268 naturally occurring contaminated shellfish samples and the stomach contents of 272 geoduck samples that had been analyzed by the standard mouse assay were tested with this new approach. Our data show excellent agreement between the ELISA data and mouse assay results. Details of this approach and results obtained from various ELISA and mouse assay studies are presented in this paper.

MATERIALS AND METHODS

Materials. Purified STX was kindly provided by Drs. E. J. Schantz (Food Research Institute, University of Wisconsin-Madison, Madison, WI) and R. W. Wannemacher, Jr. (United States Army Medical Research Institute of Infectious Diseases, Fort Detrich, MD). Neo-STX was kindly provided by Dr. Sherwood Hall of the Food and Drug Administration (Washington, D.C.) and was prepared by the method previously described (Hall et al., 1990). Bovine serum albumin (BSA; RIA

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Table 1. PSP Toxin Levels (μg/100 g) in 62 Samples of Butter Clams Analyzed by Different Methods

sample no.[a]	ELISA-A	ELISA-B	ELISA-C	A+C	A+B	mouse
945105	82	95	89	171	177	205
945194	155	361	333	488	516	1472
945195	208	109	334	541	318	1544
945196	282	110	162	445	393	1701
946192	33	14	15	48	47	32
953319	25	52	6	31	77	65
953320	23	55	7	30	78	52
953321	22	56	9	31	78	46
954884	144	11	7	151	155	137
954885	152	44	46	197	196	265
954886	572	38	55	626	609	242
Data summary: No. of samples at levels greater than 40 or 80 μg/100 g						
>40	7 (11.2)[b]	8 (12.9)	6 (9.6)	8 (12.9)	11 (17.7)	10 (16.1)
>80	7 (11.2)	4 (6.5)	4 (6.5)	7 (11.2)	7 (11.2)	7 (11.2)

[a] 51 samples contained <40 μg PSP/100 g by all the methods tested. [b] Values in parentheses indicate % of a total of 62 samples.

grade) and horseradish peroxidase (HRP; ELISA grade, cat. no. 605 220) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Tween 20 and *o*-phenylenediamine (OPD) were obtained from Sigma Chemical Company (St. Louis, MO). ELISA microwell plates were purchased from Nunc (high binding capacity, Nunc Co. no. 4-69914, Denmark). Antibodies against STX and neo-STX were prepared in our laborotory as previously described (Chu and Fan, 1985; Chu et al., 1992). The enzyme−toxin conjugates, including *STX−HRP* and *neo-STX-HRP*, were prepared via reductive alkylation methods as described by Huang et al. (1996). All chemicals and organic solvents were reagent grade or better.

Sample Treatments. To test the efficacy of different dc-ELISA protocols in the naturally contaminated samples, 1540 samples collected from various locations in Alaska were extracted with 0.18 N HCl according to the standard *Association of Official Analytical Chemists* (*AOAC*) protocols and analyzed for PSP toxins by mouse assay in the Alaska Laboratory in 1994. The acid extracts (1 g of tissue/mL) from the viscera of seven different species of shellfish and stomach contents from the geoducks were then shipped to University of Wisconsin (UW) for analysis by ELISA via Federal Express. To avoid any bias, mouse data were not supplied to the UW laboratory until all the ELISAs were done. For the ELISA, 1:400 and 1:800 dilutions with PBS for each of the acid extracts were made; the diluted extracts were then subjected to the ELISA with no further cleanup treatment. With two such dilutions, the toxin concentration of less than or equal to 80 μg/100 g of sample would fall within the standard curve range for the assay.

dc-ELISA. The protocol for the dc-ELISA was essentially the same as we previously described (Huang et al., 1996), with three different formats, including (A) coating antibodies against STX and using *STX-HRP* as a marker; (B) coating neo-STX antibodies and then using *STX-HRP* as marker; and (C) coating neo-STX antibodies and then using *neo-STX-HRP* as a marker. For coating the antibody to the solid-phase, 100 μL of the diluted antibody solution (57 μg of anti-STX or anti-neo-STX IgG/mL of 0.01 mol/L PBS, pH 7.5) were added to each well of a Nunc microwell plate and kept in a cold room overnight. In general, after incubating the coated plate at 4 °C overnight, the plate was washed with PBS-Tween (0.35 mL/well; 0.05% Tween-20 in 0.01 M PBS, pH 7.5) in an automatic ELISA washer (Dynatech model B Miniwasher) followed by incubation with BSA-PBS (0.17 mL/well; 0.1% BSA in 0.01 M PBS, pH 7.5) at 37 °C for 30 min. The plate was washed again with PBS-Tween (0.35 mL/well) four times, followed by addition of 0.05 mL of standard STX or neo-STX at different concentrations or blank buffer, or sample solution together with 0.05 mL of *STX-HRP* (0.25 μg/mL) or *neo-STX-HRP* conjugates (0.5 μg/mL) to each well. After incubation at 37 °C for 60 min, the plate was washed, and 0.1 mL of freshly prepared OPD substrate solution [10 mg of OPD plus 13 μL of 30% hydrogen peroxide in 25 mL of 0.05 M citrate-phosphate buffer (4.8 g citric acid and 7.1 g of Na_2HPO_4 in 500 mL of distilled water with pH adjusted to 5.0)] was added. Ten minutes after incubation at room temperature in the dark, the reaction was terminated by adding 0.1 mL of 1 N HCl. Absorbance at 490 nm was determined in an automatic ELISA reader (THERMO/max microplate reader, Molecular Devices Company, Menlo Park, CA). Triplicate analyses were made for each sample.

Standard STX or neo-STX solutions were prepared as stock solution A (10 μg/mL of 0.1 N HCl) and stock solution B (1 μg/mL 0.01 N HCl) and kept in the freezer. A series of standard solutions, prepared by diluting stock solution B to appropriate concentrations with PBS, was used within 1 week. Because of the instability of the toxin at neutral and alkaline pH, the diluted solution stock solution B should be never kept more than one month in the refrigerator.

Toxin concentration in each sample was determined by comparison with the standard curves for STX and neo-STX that were established by a series of toxin concentration runs under the same ELISA conditions each day (Huang et al., 1996). Five sets of ELISA data, including data from anti-STX/*STX-HRP* (ELISA-A), anti-neo-STX/*STX-HRP* (ELISA-B) and anti-neo-STX/*neo-STX-HRP* (ELISA-C) alone, the combination of ELISA-A and ELISA-C, and the combination of ELISA-A and ELISA-B were generated from the aforementioned three ELISA formats for each sample.

RESULTS AND DISCUSSION

Five sets of ELISA and one set of mouse assay data were generated for each sample, so it is impossible to present all the detailed data in the present study. Rather, data from a representative group are presented. Results of original data for the toxin levels in each of the butter clam samples as analyzed by different methods are shown in Table 1. Among 62 samples analyzed in this group, 51 samples contained either no PSP toxins or <40 μg/100 g of sample (ELISA range from 0 to 15.6 μg/100 g, data omitted). For samples containing >40 μg/100 g, toxin levels also varied considerablely. The toxins levels obtained from ELISA-A plus ELISA-B and data from ELISA-A plus ELISA-C are very close to the mouse assay data when the total toxin levels were <200 μg/100 g of sample. At levels >200 μg/100 g (e.g., samples 945194, 945195, 945196, and 954885), ELISA data were lower than the mouse assay data. This discrepancy is primarily because the ELISA data for these samples were obtained from the lower portion of the standard curve. Because the ELISA protocol in the present study was designed for use as a screening test, only two dilutions of sample extracts were made before ELISA. Thus, at high PSP toxin levels, the data were not in the linear range (0.05 to 1.0 ng/mL) of the standard curve (Huang et al., 1996). Similarly, as we reported before (Huang et al., 1996), the concentrations causing 50% inhibition of the binding of toxin−HPR conjugates to the solid-phase antibodies

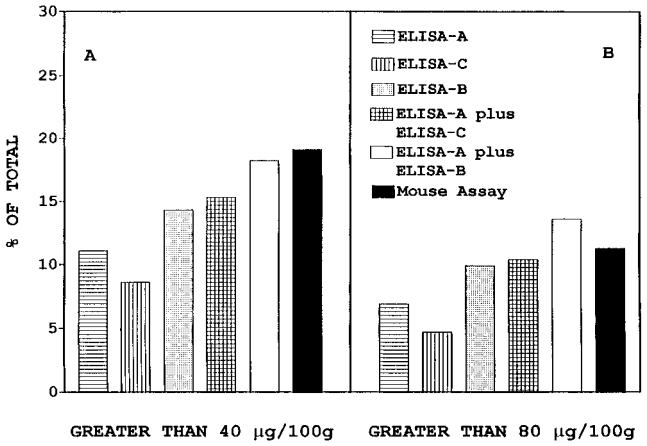

Figure 1. Population of PSP toxins contaminated with more than 40 (1A) and 80 (1B) μg/100 g of sample detected by various methods in 1540 samples.

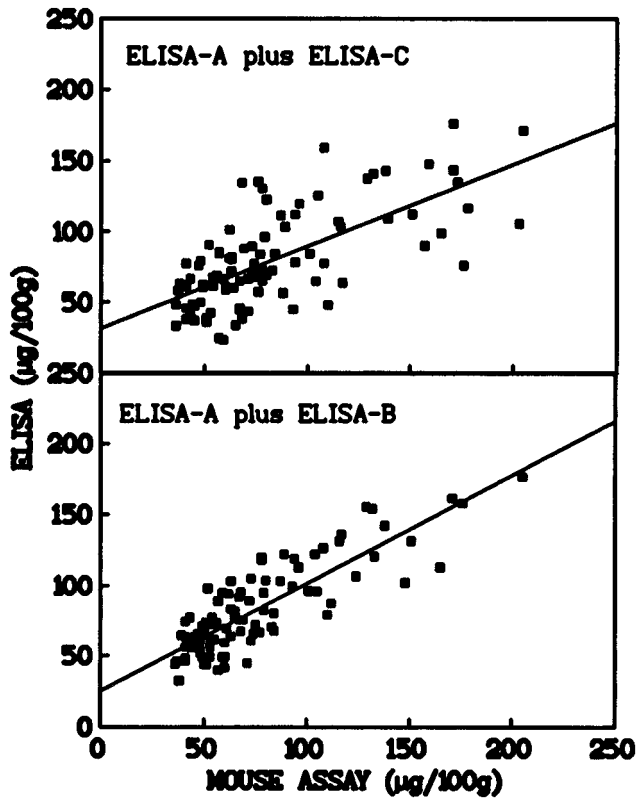

Figure 2. Correlation of total PSP toxin levels obtained from mouse assay with data from ELISA-A (anti-STX/*STX-HRP*) plus ELISA-B (anti-neo-STX/*STX-HRP*) method and ELISA-A plus ELISA-C (anti-neo-STX/*neo-STX-HRP*) method. A total of 98 samples each of which had a total ELISA value between 40 and 200 μg/100 g was selected for the statistical analysis.

in ELISA-A and ELISA-B by free STX and neo-STX were 0.28 and 0.34 ng/mL, respectively. For quantitative data, further dilution is necessary for samples containing >100 μg of toxin/100 g of sample. The overall data for the numbers of samples with >40 and 80 μg/100 g of sample are shown in the bottom portion of Table 1. These data were used in calculating the overall results presented Figures 1 and 2.

The overall results for all the samples identified as toxic with four different analyses are shown in Figure 1. Among 1540 samples analyzed, anti-STX/*STX-HRP* ELISA (ELISA-A), anti-neo-STX/*STX-HRP* ELISA (ELISA-B), anti-neo-STX/*neo-STX-HRP* ELISA (ELISA-C), combined data from ELISA-A and ELISA-C, combined data from ELISA-A and ELISA-B, and mouse assay identified 11.1, 14.3, 8.6, 15.3, 18.2, and 19%, respectively, of the total samples contaminated with >40 μg of PSP toxins/100 g of sample (Figure 1A). ELISA-A, ELISA-B, ELISA-C, combined data from ELISA-A and ELISA-C, combined data from ELISA-A and ELISA-B, and mouse assay identified 6.9, 9.9, 4.7, 10.4, 13.6, and 11.3%, respectively, of the total samples contaminated with >80 μg of PSP toxins/100 g of sample (Figure 1B).

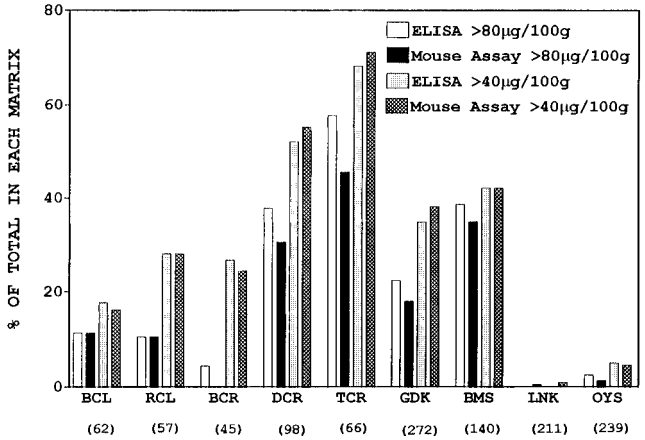

Figure 3. Population of PSP toxins in each sample matrix that contained more than 40 and 80 μg/100 g of sample as detected by mouse assay and by ELISA method. In the ELISA assay, data from the combination ELISA-A (anti-STX/*STX-HRP*) plus ELISA-B (anti-neo-STX/*STX-HRP*) were used. The total numbers in each matrix analyzed are shown in the parentheses. The abbreviations are: BCL, butter clams; RCL, razor clams; BCR, Bairdi crabs (all were negative in mouse assay); DCR, dungeness crabs; TCR, Tanner crabs; GDK, geoduck; BMS, blue mussels; LNK, little necks; and OYS, oysters.

Results for the total PSP toxin levels obtained from ELISA-A plus ELISA-B and ELISA-A plus ELISA-C and their correlation with mouse data, as analyzed with a statistical program (Prism Computer program, GraphPad Software, Inc., San Diego, CA), are shown in Figure 2. Because only two sample dilutions were made for the ELISA, only data for toxin levels of 40−200 μg/100 g (a total of 98 samples) were subjected to this analysis. A good linear correlation between mouse assay results and the total ELISA results was found. The coefficient of determination (r^2 value) between the total ELISA data from ELISA-A plus ELISA-B and mouse assay was 0.84 at $p < 0.0001$ (y [ELISA-A plus ELISA-B data] = 25.1 + 0.76 X [mouse data]). The correlation between the total ELISA data obtained from ELISA-A plus ELISA-C and mouse assay was less strong, with a r^2 value of 0.69 at $p < 0.0001$ (y [ELISA-A plus ELISA-C] = 31.5 + 0.58 X [mouse data]).

The distribution of toxins in different types of samples is shown in Figure 3. Because the overall data obtained from the combination of methods A and B was most comparable to those obtained from the mouse assay, only the data from these two approaches for each individual matrix of samples are shown. It is apparent that at both levels, tanner crabs and dungeness crabs had more toxic samples than others. In most cases, the number of samples from the combination of ELISA-A plus ELISA-C methods and from ELISA-A plus ELISA-B was similar to or slightly more than the number of the samples obtained from the mouse assay. Among all the samples tested, 360 samples extracts, including 5 red neck clams, 7 surf clams, 37 king crabs, 40 opilio crabs,

16 weathervane scallops, and 255 snails, were either identified as negative or had <40 μg of toxin/100 g of sample by mouse assay and by all of the ELISA formats tested. Except for the data obtained from ELISA-C (anti-neo-STX/*neo-STX-HRP* group), data from all four other ELISA combinations of 57 razor clams also completely agreed with the mouse assay data in identifying the toxic shellfish at the threshold level of 80 μg of toxins/100 g of samples.

Results from the present study clearly show that data from the combination of ELISA-A and ELISA-B compared most favorably with mouse assay data at both the 40- and 80-μg/100 g levels. Using the threshold level of 80 μg/100 g as the cut-off line, some false positives were identified with this approach. Although this result might be due to the inherent problems associated with the mouse assay, the ELISA data could provide a safety factor to avoid any false negatives. Another factor contributing to false positive results in the ELISA in comparison with mouse data is the cross-reactivity of the antibodies with some STX-related PSP toxins that have lower toxicity in the mouse. Data from the combination of ELISA-A and ELISA-C also agree well with the mouse data, but some false negatives were observed.

ELISA data on individual toxins apparently were not sufficient to represent the total toxins present in the samples because considerable false negatives were observed. These results are not surprising because the antibodies used in the assays are very specific, namely that anti-STX antibodies are most specific for STX with cross-reactivity with gonyautoxins, and anti-neo-STX antibodies are most spepcific for neo-STX (Chu and Fan, 1985; Chu et al., 1992; Huang et al., 1996). The overall data of the present study agree well with the data from our recent studies in which we found that combinations of data obtained from individual ELISA analyses for specific toxins correlated most strongly with mouse assay data (Huang et al., 1996).

Another advanage for simultaneous analysis of ELISA-A and ELISA-B is that only one enzyme-marker (i.e., *STX-HRP*) is needed in the assay. Because of the limited supply of neo-STX, the ELISA-C format is less attractive even though this assay could specifically determine neo-STX. To avoid two ELISAs, attempts to coat various combinations of anti-STX and anti-neo-STX in the same well were made. However, because of the complexity of cross-reactivity of the antibodies with the toxins in a mixture, no satifactory results have been obtained (Huang et al., 1996). Until we learn more about their interactions, the present format, namely coating antibodies separately and using the same *STX-HRP* as the marker, is recommended. Using the present ELISAs as the screening test, as much as 85% of the population that has been found to be contaminated by PSP toxins at or below 80 μg/100 g of the sample could avoid having to be run in the mouse assay. Quantitative information on the toxin levels in the samples could also be obtained. For samples contaminated with >80 μg toxin/100 g, additional dilution is necessary before ELISA. If 40 μg/100 g is selected as the threshold line, 80% of the population could still avoid having to be run in the mouse assay.

Conclusion. In the present study, several direct competitive ELISA protocols have been established for the analysis of both STX and neo-STX. Although each format has its own merits for determination of individual toxins, we found that an ELISA involving the use of specific antibodies against either STX or neo-STX together with a common marker, *STX-HRP*, is most versatile. The assay provides both sensitivity and specificity for the major toxins involved in the PSP incidents and could be used as a quick screening test for PSP toxins in shellfish and other marine samples.

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