Note

Evaluation of Enzyme-Linked Immunosorbent Assay (ELISA) Kit for Paralytic Shellfish Poisoning Toxins

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The reliability and utility of an enzyme-linked immunosorbent assay (ELISA) kit for paralytic shellfish poisoning (PSP) toxins were examined. Compared with the mouse bioassay, which is an official method worldwide, as described by the Association of Official Analytical Chemists (AOAC), the system showed remarkable sensitivity to saxitoxin (STX). The recovery of saxitoxin diacetate (STX-A) from clam meat was almost 100% over a certain range of the toxin concentration. However, the kit exhibited unpredictable cross-reactivities to a mixture of gonyautoxins (GTXs), and underestimated the toxicity of some naturally contaminated shellfish samples which were harvested in the sea near Japan.

We consider that the existing mouse bioassay can not be replaced with the ELISA kit for the purpose of screening inshore shellfish samples. Considering the advantages of this method, however, the kit should be available for certain uses provided that its limitations are borne in mind.

Key words: paralytic shellfish poisoning toxins, saxitoxin, gonyautoxin, ELISA, mouse bioassay

Introduction

Paralytic shellfish poisoning (PSP) toxins that act by blocking the sodium channel in neuronal and muscle cell membranes are a notorious cause of fatal food poisonings, and are therefore under world-wide surveillance. Many attempts have been made to develop new methods to analyze PSP toxins and new toxicity-detecting-systems which can take over the mouse bioassay which is currently adopted as an official method worldwide. The cultured cell assay system¹⁻⁴⁾, liquid chromatographic method⁵⁻⁸⁾, radioimmunoassay⁹⁾ and enzyme immunoassay¹⁰⁻¹³⁾ have been developed, and are known to be sensitive and time-saving compared with the mouse bioassay. However, most of these methods need expensive equipment and some have a limited detection spectrum of the toxins.

Direct enzyme-linked immunosorbent assay

(ELISA) was introduced by Usleber *et al.*¹³⁾ in 1994, and the system is now commercially available. The kit is relatively inexpensive and is convenient to use. The antibody used in this system was raised against saxitoxin (STX), which is the most potent PSP toxin, and the ELISA kit has a very high sensitivity to STX.

However, the predominant PSP toxins in the seas near Japan are not STX, but rather other toxins such as gonyautoxins (GTXs)^{14), 15)}. Because the principle of ELISA is antigen-antibody binding, the detection efficiency of an ELISA system for a toxin depends on the cross-reactivity of the antibody used in the system. The ELISA kit for STX shows variable cross-reactivity against GTXs, from less than 0.1 to 55%. Further examination of the sensitivity of this kit is needed before its practical application to Japanese inshore shellfish samples. Moreover, it remains to be clarified whether meat com-

ponents of shellfish might interfere with the detection of toxins by this system.

Here we report on the reliability of this ELISA system applied to mixtures of GTXs and to real shellfish specimens.

Materials and Methods

ELISA kit and reagents

RIDASCREEN Saxitoxin (r-Biopharm, Darmstadt, Germany) was purchased through AZmax, Chiba, Japan. Saxitoxin diacetate (STX-A) was purchased from Calbiochem, San Diego, U.S.A. STX-A was dissolved and diluted to suitable concentrations in a solution which was adjusted to pH 3 with 0.1 N HCl and 0.1 N NaOH.

Two kinds of mixtures of gonyautoxin 2 and 3, GTX2/3-A and GTX2/3-B, were generous gifts from Prof. Tamao Noguchi, Faculty of Fisheries, Nagasaki University. The composition of GTX2/3-A was GTX2:GTX3 (4.68:1) and that of GTX2/3-B was GTX2:GTX3 (1:24.75).

Shellfish samples

Toxic ormers which had been imported from Spain and toxic scallops harvested in the Hokkaido area were also kindly presented by Prof. Tamao Noguchi. Non-toxic clams were purchased at a market and the lack of toxicity was confirmed by mouse bioassay. Acid extraction from shellfish and the mouse bioassay were performed following the official method in Japan¹⁶⁾.

To estimate the recovery of STX-A from shellfish, 1 μ mol/ml of STX-A solution was added to the homogenate and to the acid extract of non-toxic clams to make 2 and 1 μ g/ml, respectively. The contaminated homogenate was subjected to the usual extraction procedure, and the resulting extracts were serially diluted with pH 3 solution so that the final STX-A concentrations were 10, 30, 90, 270 and 810 pg/ml.

For the ELISA assay of naturally contaminated shellfish samples, acid extracts were diluted to 1:100, 1:500, 1:2500 and 1:12500. ELISA assay was performed for each diluted sample in duplicate, and the data were converted to STX equivalent. Since the standard curve was linear between 30 and 310 pg/ml of STX, only data which fell within this range were used for the final calculation of the toxin con-

centration.

Mice

Male SPF ddY mice (4-week-old, 19 to 21 g B. W.) were purchased from SLC Japan, Shizuoka, Japan. The adopted CF value was 0.19 μ g/MU¹⁷⁾.

Procedure of ELISA

The principle of the kit is the competition of free and enzyme-labeled toxin for the antibody binding sites.

According to the instructions with the ELISA kit, 50 μ l of STX standard or sample solution was allowed to react with the coated antibodies in competition with 50 μ l of STX-enzyme complex solution for 60 min at room temperature in the wells of microtiter strips. After a thorough wash of the wells, 50 μ l aliquots of substrate and chromogen solution were added and incubation was continued for a further 30 min at room temperature in the dark. The reaction was stopped by adding 100 μ l of stop reagent, and OD₄₅₀ was measured for each well. The absorption is inversely proportional to the saxitoxin concentration in the standard or the sample. It is possible to assay as many as 18 samples within 2 hours. Percent maximum value was calculated by use of the equation:

$$(\text{OD}_{450} \text{ of standard or sample} / \text{OD}_{450} \text{ of } 0 \mu\text{g/ml STX}) \times 100.$$

Results and Discussion

Sensitivity of ELISA to STX and recovery of toxin from shellfish samples

A standard curve was reproducibly obtained when standard solutions of the kit and STX-A were used. The detection limit for STX concentration in shellfish calculated from the standard curve was 2 ng/g tissue. This value is 1/200 of that of the mouse bioassay.

STX-A concentrations were assayed for serially diluted clam samples spiked with STX-A, as described above. The recoveries of STX-A were between 99.5 to 105.1% in the range of 90 to 810 pg/ml, and between 89.8 to 111.3% in the range of 10 and 30 pg/ml. We concluded that components of shellfish, whether before or after the extraction, do not affect the detection of STX-A by this ELISA system to any

significant degree.

Analysis of GTX2/3 by the ELISA system

Next we examined the sensitivity of the ELISA kit to GTXs using two kinds of GTX mixture, GTX2/3-A and GTX2/3-B. For GTX2/3-A the composition was GTX2:GTX3 (4.68:1) and for GTX2/3-B the composition was (1:24.75). The present kit was as reactive to GTX2/3-A as it was to STX-A, but was not nearly so reactive to GTX2/3-B, with the result that the toxin content was grossly underestimated (Fig. 1). The toxin concentrations for STX-A, GTX2/3-A and GTX2/3-B which gave 50% inhibition of the binding of STX-enzyme complex to the kit antibodies were 71.9, 81.0 and 725.9 pg/ml, respectively.

The instruction manual describes the relative cross-reactivity to individual GTX2 and GTX3 as about 30 and 55%, respectively, but it appears that when GTX2 and GTX3 are mixed, as often happens in nature, the resulting reactivity to the kit and that predicted from the actual ratio of each toxin will be different. Since the reason for this observation remains unknown, further investigations seem to be needed. Usleber *et al.*¹³⁾ have stated that the cross-reactivity to a GTX2/3 mixture of direct ELISA was large enough to detect the regulatory level of PSP

toxins in shellfish. They did not, however, clarify, the ratio of GTX2 and GTX3 in the mixture used. In view of our data, it is possible that, depending on the ratio of GTX2 and GTX3, the real toxicity of their sample was underestimated. To make matters worse, the cross-reactivity of this kit to individual GTX1 and GTX4 is less than 0.1%. When this kit is applied to GTX-rich shellfish samples, the presence of the toxin, even if it is above the regulatory level, might be overlooked. We next examined this possibility using real samples.

Assay of shellfish with the ELISA kit and by the mouse method

The toxicity of muscle of naturally contaminated ormer and hepatopancreas of toxic scallops was tested both by the mouse bioassay and by the present ELISA kit. The values were converted into and expressed as concentration of saxitoxin equivalent (Table 1). By the ELISA system, the toxicity of ormer was estimated at more than three times that in the mouse assay. On the other hand, compared with the toxicities determined by the mouse assay, those of scallop samples estimated by the ELISA kit were 53.5 and 49.7%. The Spanish ormers were contaminated by STX group compounds such as decarbamoyl STX¹⁸⁾, but Japanese scallops contained the GTX group^{14), 19)}. Since this ELISA was far more sensitive to STX than the mouse bioassay, the result on ormer is reasonable. As had been suspected, the toxicities of scallop samples were underestimated by this kit. Scallop sample 2, whose toxicity was determined as positive ($>80 \mu\text{g}/100 \text{ g}$) by the mouse bioassay, was borderline as determined by this kit (Table 1).

The variation in cross-reactivity against PSP toxins is clearly important when this kit is applied to real shellfish samples, especially to those from the seas near Japan. In practice, it seems impossible to replace the present mouse bioassay completely with this kit. Nevertheless, the high sensitivity to STX, short analysis time per sample, convenience in handling, and most of all, lack of a requirement for experimental animals are great advantages of this kit. It might be worth while making use of this system, for example, in the screening of imported shell-

Fig. 1. The sensitivity of the ELISA kit to GTX mixtures

--○--: STX-A, —▲—: GTX2/3-A (GTX2: GTX3=4.68:1), —■—: GTX2/3-B (GTX2: GTX3=1:24.75). Fifty μl of diluted toxin was applied to the well of the ELISA kit and processed as described in the text.

Table 1. The Toxicities of Naturally Contaminated Shellfish Samples Assayed by the ELISA Kit and the Mouse Bioassay

Sample	PSP concentration (μ g STX-eq./100 g meat)	
	ELISA	Mouse bioassay
Ormer	766	227
Scallop 1	21.2	39.6
Scallop 2	85.9	173

Values are based on the means of duplicate assays of serially diluted samples giving values between 30 and 310 pg/ml as saxitoxin equivalent.

fish which are presumed to be contaminated mainly by STX group compounds, or in periodical testing during PSP-positive seasons to detect the start of the decline of toxicity.

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