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Analytical Methods

A highly rapid and simple competitive enzyme-linked immunosorbent assay for monitoring paralytic shellfish poisoning toxins in shellfish

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ABSTRACT

Using a streptavidin-coated well plate, a biotin-labelled anti-gonyautoxin 2/3 monoclonal antibody GT-13A, and a decarbamoyl saxitoxin-peroxidase conjugate, a direct competitive enzyme-linked immunosorbent assay (PSP-ELISA) was developed for monitoring paralytic shellfish poisoning (PSP) toxins in shellfish. This assay is simple to perform and can be completed in approximately 20 min. The PSP-ELISA was compared to the mouse bioassay (MBA) for the detection of PSP toxins in shellfish samples ($n = 83$) collected from the coast of Osaka Prefecture, Japan. When positive and negative results were indicated based on the regulatory limit for PSP toxins (4 mouse unit (MU)/g of shellfish meat), the PSP-ELISA results showed a sensitivity of 100% (25 of 25) and a specificity of 89.7% (52 of 58 samples) compared to the MBA results. These results suggest that the PSP-ELISA could be used as a rapid and simple screening method prior to the MBA.

1. Introduction

Paralytic shellfish poisoning (PSP) is one of the most potent naturally occurring seafood poisonings. PSP toxins consist of more than 20 toxin analogues, such as saxitoxin (STX), neosaxitoxin (neoSTX) and gonyautoxin (GTX) components (Fig. 1), each with a different toxicity factor (Liewellyn, 2006; Wiese, D'Agostino, Mihali, Moffitt, & Neilan, 2010). Marine dinoflagellates such as *Alexandrium catenella* and *Alexandrium tamarense* produced the PSP toxins, which then accumulate in shellfish after they utilise the dinoflagellates as a food source (Asakawa, Miyazawa, Takayama, & Noguchi, 1995). Consumption of the toxin-contaminated shellfish by humans may cause paralytic poisoning and, occasionally, death.

In many countries, the regulatory limit for PSP toxins in shellfish has been established as 800 μg of saxitoxin (STX) equivalents/kg of shellfish meat or 4 mouse unit (MU) of PSP toxins/g of shellfish meat. One MU is defined as the toxin dose of toxin required to kill a 20 g mouse in 15 min and is equivalent to 0.18 μg of STX (Chu & Fan, 1985). The mouse bioassay (MBA) standardised by the Association of Analytical Communities (AOAC) (Hollingworth & Wekell, 1990) has been accepted internationally as the official method for the quantitative measurement of the PSP toxins in shellfish. The MBA has been used to protect human health for decades. However, there are ethical, cost, time, and labour concerns associated with the use of this animal assay, especially when processing a large number of samples. A simple, rapid, cheap and reliable screening method is needed as an alternative to the MBA.

Some alternative analytical methods have been developed, e.g., high performance liquid chromatography (HPLC) with fluorescence detection (Lawrence, Niedzwiadek, & Menard, 2005; Oshima, 1995). One of these methods has been adapted by the AOAC as an alternative (AOAC, Official Method 2005.06, 2006) for the detection of PSP toxins in shellfish and has been approved by the European Union (EU) as a first action method. HPLC methods are specific and sensitive. However, they are time-consuming and expensive and requires highly trained personnel and the use of certain certified analytical PSP toxin reference standards. These standards, which are used for the correct identification and quantification of PSP toxin, are difficult to obtain. Therefore, HPLC methods are more suitable for confirmatory analysis than for screening.

Immunology-based methods that include enzyme-linked immunosorbent assays (ELISAs) (Burk, Usleber, Dietrich, & Martlbauer, 1995; Campbell et al., 2009; Chu, Hsu, Huang, Barrentt, & Allison, 1996; Chu, Huang, & Hall, 1992; Huang, Hsu, & Chu, 1996; Kawatsu, Hamano, Sugiyama, Hashizume, & Noguchi, 2002; Usleber, Dietrich, Martlbauer, & Terplan, 1994; Chu & Fan, 1985) and surface plasmon resonance biosensor (SPR) assays (Campbell et al., 2007; Campbell et al., 2009; Campbell et al., 2010; Fonfria et al., 2007; Haughey et al., 2011; Rawn, Niedzwiadek, Campbell, Higgins, & Elliott, 2009; Van den Top

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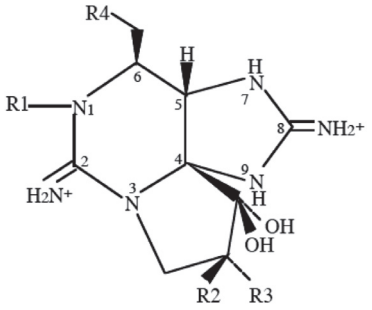

			Carbamate toxins	N-Sulfocarbamoyl toxins	Decarbamoyl toxins	Deoxydecarbamoyl toxins
R1:	**R2:**	**R3:**	**R4:** $OCONH_2$	**R4:** $OCONHSO_3^-$	**R4:** OH	**R4:** H
H	H	H	STX	GTX5	dcSTX	doSTX
H	H	OSO_3^-	GTX2	C1	dcGTX2	doGTX2
H	OSO_3^-	H	GTX3	C2	dcGTX3	doGTX3
OH	H	H	neoSTX	GTX6	dcneoSTX	
OH	H	OSO_3^-	GTX1	C3	dcGTX1	
OH	OSO_3^-	H	GTX4	C4	dcGTX4	

Fig. 1. Chemical structure of PSP toxins.

et al., 2011) have been developed for the detection of PSP toxins in shellfish. These methods are simple, rapid, and sensitive and thus appear to be more suitable than HPLC for screening toxin-contaminated shellfish. SPR assays permit high throughput and automated detection (Campbell et al., 2009; Haughey et al., 2011). However, SPR assays have high start-up costs because most laboratories do not have the expensive equipment necessary for SPR assays. In contrast, Laboratories are usually prepared to perform ELISAs for the detection of various chemical and biological compounds. Therefore, compared with SPR assays, ELISAs can be performed at a lower cost. However, compared with SPR assays, conventional ELISAs lack simplicity and rapidity.

The purpose of the present study was to develop an ELISA system that is more simple and rapid than the conventional ELISAs. Using a commercially available streptavidin-coated well plate, a biotin-labelled anti-GTX 2/3 monoclonal antibody GT-13A (biotin-GT-13A), and a decarbamoyl STX-peroxidase conjugate (dcSTX-POD), a direct competitive ELISA for the detection of PSP toxins (PSP-ELISA) was successfully developed. We report the development of the PSP-ELISA and the evaluation of its utility as a tool for screening for toxin-contaminated shellfish.

2. Materials and methods

2.1. Materials

Gonyautoxin 2/3 mixture (GTX2/3), GTX1/4, decarbamoyl GTX2/3 (dc-GTX2/3), *N*-sulfocarbamoyl GTX2/3 (C1/2) and neosaxitoxin (neoSTX) were kindly supplied by the Fisheries Agency of Japan. Decarbamoyl STX (dcSTX) was kindly provided by Dr. Yasukatsu Ohshima (Tohoku University, Sendai, Japan). Sodium periodate ($NaIO_4$), sodium borohydride ($NaBH_4$) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Peroxidase (POD; EIA grade) was from Roche Diagnostics (Indianapolis, IN), and the PD-10 column was from Amersham Pharmacia Biotech (Uppsala, Sweden). Sodium chloride (NaCl), potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), sodium carbonate, sodium hydrogen carbonate, sodium acetate, Tween 20, acetic acid, hydrochloric acid (HCl) and sulphuric acid (H_2SO_4) were from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Streptavidin-coated well plates (8-well strips, 96-well/plate), Sulfo-NHS-LC-LC-biotin and chromogenic substrate solution (1-Step™ Ultra TMB-ELISA) were from Thermo Scientific Inc. (Rockford, IL). Phosphate-buffered saline solution (PBS) was 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 in 1 L of distilled water, pH 7.4. PBS-T solution was PBS supplemented with 0.05% v/v Tween-20. The anti-GTX 2/3 monoclonal antibody GT-13A was produced in our laboratory (Kawatsu et al., 2002).

2.2. Preparation of dcSTX-POD

A dcSTX-POD conjugate was prepared according to the reductive alkylation method after oxidation of POD with $NaIO_4$ (Wilson & Nakane, 1978). Briefly, 10 mg POD in 2 mL distilled water was combined with 0.5 mL 0.1 M $NaIO_4$ (in distilled water) at 25 °C for 20 min. The reaction mixture was then loaded onto a PD-10 column, which was equilibrated with 0.1 M sodium acetate buffer (pH 4.4). After elution, the protein-containing portions (3.5 mL) were collected. Sodium carbonate buffer (0.66 mL of 1.0 M; pH 9.6) and 0.6 mL dcSTX solution (100 μg/mL in 0.1 M acetic acid) were added to 1.05 mL of the solution. After 240 min incubation at 25 °C, 90 μL of $NaBH_4$ solution (4 mg/mL in distilled water) was added and the solution was incubated at 4 °C for 30 min. The mixture was then loaded onto a PD-10 column, which was equilibrated with 0.01 M sodium phosphate buffer containing 0.15 M NaCl (pH 6.0). After elution, 0.5 mL of 16% w/v BSA (in distilled water) was added to the protein-containing portions (3.5 mL). The mixture (dcSTX-POD; 750 μg/mL) was stored at −20 °C until use.

2.3. Preparation of biotin-GT-13A

The GT-13A antibody was labelled with NHS-LC-LC-biotin according to the manufacturer's instructions (Thermo Scientific). Briefly, 27 μL of 10 mM Sulfo-NHS-LC-LC-biotin (in distilled water) was added to 1 mL of the GT-13A antibody solution (2 mg/mL in PBS). After incubation on ice for 120 min, the reaction mixture was loaded onto a PD-10 column, which was equilibrated with PBS. After elution, the protein-containing portions (3.5 mL) were collected and diluted to 4 mL with PBS. A 0.5 mL aliquot of the solution was then added to 9.5 mL of 2% w/v BSA (in PBS), and