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RESEARCH PAPER

Protein Phosphatase Inhibition Assay for Detection of Diarrhetic Shellfish Poison in Oyster

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Abstract: A method based on protein phosphatase enzyme activity inhibition for monitoring diarrhetic shellfish poison (DSP) was used to analyze the DSP toxicity in three oyster samples. Based on the standard dose–effect curve developed with a series of okadaic acid (OA) standard solutions, the DSP toxicity of the three oyster samples collected was screened, and the results showed that OA and dinophysis toxins (DTXs) were not detected in the samples without hydrolyzation. However, the OA toxicity was detected in two of the hydrolyzed samples; the concentration of the OA was 1.81 and 1.21 μg OA eq./kg oyster, respectively.

Key Words: Diarrhetic shellfish poison (DSP); Okadaic acid (OA); Protein phosphatase inhibition assay; Oyster

1 Introduction

Phycotoxins derived from the harmful algal blooms (HABs) have caused world-wide public concern in the last several decades due to their potential threat to the health of humans. Diarrhetic shellfish poison (DSP), consisting mainly of okadaic acid (OA) and dinophysis toxins (DTXs), is responsible for some familiar food poisoning incidents. The symptoms caused by consumption of DSP-contaminated shellfishes include diarrhoea, vomiting, and abdominal pain. Although no serious acute toxicities of OA and DTXs were reported, the chronic effects of the toxins, such as tumor promotion resulting from the inhibition of protein phosphatase, caused much concern.

So far many methods have been developed for the analysis of DSP toxins or toxicity because of the wide distribution of the toxins, especially in Asia and Europe. Every method used for analysis of DSP toxins, such as the mouse bioassay, enzyme-linked immunosorbent assay, cell toxicity assay, protein phosphatase inhibition assay, high-performance liquid chromatography–fluorescence detection (HPLC–FD) and HPLC–mass spectrometry (MS), has some kind of advantages and disadvantages. The mouse bioassay and HPLC methods are the most popular methods. However, the mouse bioassay method was often questioned in the last several years, because of many disadvantages associated with this method, such as the complex process in toxin extraction, the long time needed to get the result, the high detection limit (200 μg OA eq./kg, similar to the control criteria of seafood safety), and the false-positive results caused by many different reasons. The application of HPLC was often hampered by the expensive instrument and the lack of standards and expertise. Therefore, there is a need for the application of a new method in toxin and toxicity assay. One of the most promising methods that appeared recently for analysis of DSP toxins was the protein phosphatase (PP1 or PP2A, for example) inhibition assay, which was based on the inhibition of the enzyme activity by OA and DTXs. PP1 and PP2A were the major forms of protein phosphatase, which catalyzed the phosphorylation on serine and threonine residues. The inhibition of the protein phosphatase activity by OA and DTXs led to acute toxic effects like diarrhea, and chronic toxic effects such as tumor promotion. These characteristics of OA and DTXs were then used to test the toxicity of DSP-contaminated shellfish samples, using substrates that produced colorful and fluorescent products after the reaction

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was catalyzed by PP1 or PP2A. This method showed many advantages in routine assessment of DSP toxicity in shellfish samples [1–3].

To protect the health of consumers, many countries have developed criteria for seafood safety targeted on DSP contamination in shellfish products. In China, the quality of shellfish products imported and exported was inspected by the State Administration for Import & Export Commodity Inspection and Quarantine. The mouse bioassay method was used for this purpose. Recently, oysters from some aquaculture corporations in Weihai, Shandong province, were rejected because of the detection of DSP toxicity by the mouse bioassay method. Three oyster samples rejected after mouse bioassay were sent to our laboratory for determining the toxin components. The toxicity of the samples was tested using PP2A protein phosphatase inhibition assay, referred to the method used in the inter-laboratory calibration organized by Dr. Michael Quilliam (Institute for Marine Biosciences, National Research Council of Canada). The toxin of oyster samples was also analyzed by LC–MS.

2 Experimental

2.1 Reagents and apparatus

Reagents used in this experiment were all Analytical Reagent (AR) Grade or above. Sodium hydroxide, calcium chloride ($CaCl_2·2H_2O$), Tris chloride, and bovine serum albumin (fraction V) were from AMRES Co. Nickel chloride ($NiCl_2·6H_2O$), 4-methylumbelliferyl phosphate, and 4-methylumbelliferone were from Sigma. Other reagents, including hydrochloric acid, acetic acid, and sodium cholate were products of China. Water used in this experiment was purified by Millipore water purification system. HPLC Grade acetonitrile, methanol, chloroform, *n*-hexane, and ether were from Dima Technology, Inc. Protein phosphatase 2A (10 U/ml) isolated from human red blood cells as the heterodimers of 36 and 60 kDa was from Upstate Biotechnology, Inc. It was stored frozen at −18°C in 0.1 ml aliquots prepared according to the manufacturer's instructions. OA standard was purchased from Marine Analytical Chemistry Standard Program, Institute for Marine Biosciences, Canada. DTX_1 standard was purchased from Wako Pure Chemical Industries Ltd.

The experiment was carried out with a microplate reader (TECAN SAFIRE, Austria). OA and DTX_1 were analyzed with the Thermo-Finnigan HPLC–MS system that consisted of Surveyor quaternary pump and autosampler, and Finnigan LCQ^{TM} Deca XP Plus mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Finnigan, San Jose, CA, USA).

2.2 Preparation of oyster samples

Homogenized oyster samples sent to our lab were tested in this experiment. The process for sample preparation and analysis referred to the method used in the inter-laboratory calibration of the PP2A protein phosphatase inhibition assay and that in Mountfort *et al*.[4], with some modification. About 2 g (wet weight) oyster sample was extracted with 5 volumes of (1:5, w/v) 80%(v/v) methanol, with ultrasonic treatment at 200 W for 1 min. After centrifugation at 6,000 rpm for 5 min, the supernatant was collected and stored in the refrigerator. The precipitate was re-extracted twice with the same procedure described above. The supernatant collected was then combined and degreased twice with 5 ml hexane each time. The hexane was collected and combined, which was used to analyze the esters of OA and DTXs after hydrolyzation, as described below. The solution of 2 ml 0.2% acetic acid was added into the methanol phase, and the acidified methanol was extracted twice with 4 ml chloroform each time. The chloroform was combined and dried by N_2 flow under darkness. The residue was re-dissolved by 500 μl methanol and stored at −18°C under darkness.

The hexane phase collected was dried and the residue was re-dissolved in 100 μl 0.5 M sodium hydroxide solution (dissolved in 90% methanol). The mixture was kept at 75°C in a water bath for 40 min. The hydrolyzed solution was then dried, and the residue was re-dissolved in 300 μl 0.5 M hydrochloric acid solution. The solution was extracted by 300 μl ether three times. The combined ether was evaporated, and the residue was dissolved in 250 μl 80% (v/v) methanol solution. After degreased with 250 μl hexane two times, the solution was added with 100 μl 0.2% acetic acid, and the mixture was extracted twice with 400 μl chloroform each time. The chloroform was then combined and evaporated, and the residue was dissolved in 500 μl methanol and stored at −18°C under darkness.

2.3 Preparation of standard calibration curve with okadaic acid

A series of OA standard solutions, 0.03, 0.15, 0.3, 0.75, 1.5, 3, 7.5, 15, 30, 100, 300, and 3,000 nM, was prepared in 16% methanol solution. The standard solutions were used to prepare a calibration curve in PP2A protein phosphatase inhibition assay at the range from 0.03 to 3,000 nM. Triplicate standard solutions were used during the assay. The assay was carried out in 96-well plates with a microplate reader (TECAN SAFIRE, Austria). The reaction system contained 10 μl Tris buffer (50 mM, pH 7.0), 5 μl $NiCl_2$ (40 mM), 35μl bovine serum albumin (10 mg/ml in Tris buffer), 10 μl sodium cholate (6.7 mM), 10 μl enzyme (0.02 U per assay), 10 μl standard solution, and 120 μl fluorescent substrate 4-methylumbelliferyl phosphate (MUP) (1.7 mM). The reaction was started by the injection of substrate solution,