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Assays and Analytical Methods for Determining Lipophilic Shellfish Toxins

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ABSTRACT

Worldwide, bivalve mollusks would accumulate toxins when toxic plankton proliferate in the environment and thus cause food poisoning. Based on their solubility and toxic natures, the toxins are separable into two classes: water-soluble toxins named paralytic shellfish toxins (PSP toxins) and amnesic shellfish toxins (ASP toxins = domoic acids), respectively, and other toxins soluble in organic solvents (lipophilic toxins). Because of the difficulty to cover the wide range of the toxins, this presentation mainly deals with the topics related to the lipophilic toxins.

There are three types of poisoning that have actually taken place after eating shellfish; diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), and azaspiracid poisoning (AZP). The causative toxins in DSP are okadaic acid (OA), dinophysistoxin-1 (DTX1 = 35-methylokadaic acid), 7-*O*-acylOA, and 7-*O*-DTX1: those in NSP are brevetoxins-B1 through -B4 (BTXB1~B4); and those in AZP are azaspiracids-1 through 5. Additionally, there are toxins that frequently occur in shellfish but have not been proven to cause human poisoning. The polyether macrolide toxins named pectenotoxins (PTX) occur together with OA and DTX1 in scallops. The toxicity of PTXs by intraperitoneal (i.p.) injection is strong but their toxicity by oral route is much less significant. Unlike scallops mussels hydrolyze PTXs to nontoxic seco acids. Yessotoxins (YTX) also occur widely in mussels and scallops. By i.p. injection YTX is one of the most potent lipophilic toxins but orally it is far less toxic, casting a doubt about its actual involvement human intoxication.

Currently, monitoring of shellfish toxicity is being implemented in many countries by using mouse bioassays. Typically, shellfish hepatopancreas are extracted with acetone, the acetone extracts are evaporated, the residue is partitioned between diethyl ether and water, and the residue from the diethyl ether is injected (i.p.) into a mouse. Various concentrations of test solutions are to be used for injection and the mice should be observed for survival

in 24 h. The mouse bioassays can be carried out in any places where mice are available for use and do not require costly instruments. However, the method can not specify the toxins that lead to the death of mice and is morally discouraging because of killing a large number of mice. In order to avoid the problems inherent to animal assays, we have developed an enzyme inhibition assay for the DSP toxins, an ELISA assay for the YTXs, and an LC-MS method for determining fourteen representative toxins.

The representative DSP toxins, OA and DTX1, strongly, selectively, and dose-dependently inhibit serine/threonine protein phosphtase 2A (PP2A). Because the enzyme can also hydrolyze colorless *p*-nitrophenol phosphate (pNPP) to brown-colored *p*-nitrophenol, the extent of enzyme inhibition (= inhibitor amounts) is easily quantified by measuring the degrees of the color formation. Preceding efforts were made by different research groups to quantify PP2A-inhibitors based on this scheme. However, these attempts did not succeed mainly because of poor reproducibility of the results. The problems seemed to have derived from the fluctuating quality of the enzyme used, which was a dimeric polypeptide consisted of structural and catalytic subunits and isolated from biological material. We inferred that the catalytic subunit should be less vulnerable to structural changes during isolation process and decided to test a catalytic subunit prepared from the marine snail *Neptunea arthritica*. The catalytic subunit showed a high sensitivity and stability and enabled us to construct a rapid and sensitive kit for assaying OA and DTX1. The assay results obtained by the kits showed a good agreement with the results obtained by LC-MS analysis. Though the acyl esters of OA and DTX1 do not inhibit PP2A, they could be easily determined after hydrolysis in an HCl solution.

In certain regions in Japan, Scandinavia, and some other countries, YTX and its analogs accumulate in shellfish frequently and often persistently, making it a priority of the regions to monitor these toxins. We prepared polyclonal anti-YTX antibodies in two ways. First, YTX was treated with ozone to produce an aldehyde function in the side chain. In the second experiment, we chose a YTX metabolite, carboxy-YTX. The aldehyde-YTX and carboxyYTX were conjugated respectively to carrier proteins and the conjugates were used to immunize rabbits. The polycolonal antibodies obtained showed higher reactivity toward YTX metabolites than to YTX itself, regardless of the type of the antigen used for immunization. We constructed a proto type ELISA kit for assaying YTXs and carried out a validation study using naturally contaminated shellfish extracts. The ELISA kits produced much higher results than those produced by LC-MS analysis, but there was a linear correlation between the data of the two methods. It was thus demonstrated that the ELISA kits would be useful for monitoring the YTXs accumulated in shellfish.

As mentioned first in this presentation, there are a number of different types of lipophilic toxins that are difficult to distinguish by the mouse assay method. Precise quantification of the toxins is also difficult to achieve by the mouse assay. In theory the LC-MS methodology would enable identification and quantification of the toxins with a high precision. The largest obstacle in the method development is the lack of standard toxins to be used as references in the analysis. With a great effort we produced 14 standard toxins representative for the DSP, NSP, AZA, PTX, and YTX. Using these standards we optimized the LC-MS conditions. All the 14 toxins either spiked to tissue homogenates or present in naturally contaminated tissues could be quantitatively determined in a single run of 30 min. The highly effective performance of the LC-MS method was thus demonstrated.

EDUCATION

1957	Marine Biochemistry, University of Tokyo B.A.
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1966	Marine Biochemistry, University of Tokyo Marine Biochemistry Ph.D.

EXPERIENCE

1992–1998	Bioorganic Chemistry, Tohoku University
1998	Prof. Emeritus, Tohoku University
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