Rapid Paper

Fluorometric Measurement of Yessotoxins in Shellfish by High-pressure Liquid Chromatography

Takeshi YASUMOTO and Azusa TAKIZAWA

Faculty of Agriculture, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981, Japan
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A rapid HPLC method with fluorescence detection of yessotoxin (YTX) and its two analogs (45-OHYTX and norYTX) in mussels and scallops is presented. A dienophile reagent, DMEQ-TAD, was used for fluorescence labeling. YTX was measured in the range 1–100 ng. The method confirmed the occurrence of YTX and 45-OHYTX for the first time in mussels from Chile and New Zealand.

Key words: fluorometric measurement; DMEQ-TAD; yessotoxin; HPLC; mussels

Worldwide infestation of bivalve shellfish by microalgal toxins poses serious threats to both human health and shellfish industries. Yessotoxin (YTX, Fig. 1) is one of such toxins found in Japan and Norway, together with dinophysistoxin-1 (DTX1) or okadaic acid (OA), which cause diarrheal shellfish poisoning (DSP). More recently, 45-hydroxyyessotoxin (45-OHYTX, Fig. 1) and 45,46,47-trinoryessotoxin (norYTX, Fig. 1) were identified, and the absolute configuration of YTX was reported. Structurally, YTX differs from other DSP-toxins but resembles the brevetoxins and ciguatoxins in having a ladder-shaped polycyclic ether skeleton. Unlike DTX1 and OA, YTX shows neither diarrheagenicity nor inhibition of protein phosphatase 2A. The dinoflagellate Dinophysis fortii, which produces DTX1 and OA, has been presumed not to produce YTX. Accordingly, arguments existed as to whether or not to include YTX in the DSP category. However, the lack of a proper method of measurement for YTXs has made it difficult to distinguish them from other diarrheic toxins. Because the YTXs have a conjugated diene in the side-chain, we thought that they would yield fluorescent adducts with a dienophile reagent, DMEQ-TAD.

In this paper, we report a method for measuring YTX, 45-OHYTX, and norYTX by using DMEQ-TAD and high-pressure liquid chromatography (HPLC). Examples of its successful application to toxic shellfish are also reported.

Materials and Methods

Materials. Scallops (P. yessoensis) and mussels (M. edulis) used in control and recovery tests were purchased at a food market in December, when shellfish were known to be free of toxins. Their digestive glands (DG) were further confirmed to be nontoxic by bioassays on mice. The following shellfish samples were tested for YTX: scallops (P. yessoensis) collected in Mutsu Bay, Japan, in June 1995; blue mussels (M. edulis) collected in Miyagi Prefecture, Japan, in July 1996; mussels (M. chilensis) collected at the Chones Archipelago in Chile in January 1991; and an acetone extract prepared by the Cawthron Institute from greenshell mussels (Perna canaliculus) collected at the Colomandel Peninsula, New Zealand, in January 1993. These shellfish samples had been proven by local officials to be toxic by mouse assays. Because the toxins were localized in DG, only this organ was used for analysis. Standard YTX, 45-OHYTX, and norYTX were prepared as described previously.

Reagents. MoH, Mn(II), CHCl₃, Na₂HPO₄·12H₂O, and NaH₂PO₄·2H₂O were of reagent grades and used as received. The fluorogenic reagent, DMEQ-TAD, was purchased from Wako Pure Chemical Industries (Osaka) and used as 0.1% solution in CH₂Cl₂. Sep-pak plus C₁₈ short body cartridge columns (Waters, U.S.A.) were washed with 6 ml of 20 mM phosphate buffer (pH 5.8) before equilibration with solvents to be used. Phosphate buffer stock solution was prepared by adding a 200 mM solution of Na₂HPO₄ to a 200 mM solution of NaH₂PO₄ until it reached pH 5.8, followed by filtration through a membrane filter (0.2 µm, Advantec Toyo).

Reaction of YTX with DMEQ-TAD. In a preparative scale experiment, 50 µg (42 nmol) of YTX was reacted with 58 µg (168 nmol) of DMEQ-TAD in 50 µl of CH₂Cl₂, at room temperature for 2 h. The solvent was evaporated under an N₂ stream, and the DMEQ-TAD adduct of YTX (DMEQ-TAD-YTX) in the residue was purified on a Cosmosil SC18AR column (4.6 x 250 mm, Nacalai Tesque, Kyoto) with MeOH/H₂O (8:2). Because the reaction yielded a mixture of (C-42) epimers (Fig. 2), each epimer was isolated by being confirmed by ESIMS. For analytical scale reaction, brown colored micro-tubes (8 x 50 mm) with polysyntene caps were used. To a micro-tube containing 1 to 100 ng of YTX was added 0.1% solution of DMEQ-TAD in CH₂Cl₂ (50 µl), and the mixture was kept at room temperature for 2 h. After the solvent was evaporated, the reaction products were dissolved in 50 µl MeOH and 10 µl was injected into the HPLC system.

Extraction of YTX from digestive glands of shellfish. One gram of DG homogenate prepared from scallops or mussels was extracted with 9 ml of MeOH/H₂O (8:2) in a homogenizer for 3 min. The homogenate was centrifuged at 3000 rpm for 10 min. A 0.5 ml portion of the supernatant was mixed with 1.5 ml of 20 mM phosphate buffer (pH 5.8) and put onto

Fig. 1. Structures of Yessotoxins.

To whom correspondence should be addressed (TEL: 022-717-8814; FAX: 022-717-8817).

Abbreviations: YTX, yessotoxin; 45-OHYTX, 45-hydroxyyessotoxin; norYTX, 45,46,47-trinoryessotoxin; DMEQ-TAD, 4-[(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinazolin-6-yl)ethyl]-1,2,4-triazoline-3,5-dione; DG, digestive glands; DSP, diarrheic shellfish poisoning.
a Sep-pak column. The column was washed with 6 ml of MeOH/H₂O (2:8), and the YTX was eluted with 6 ml of MeOH/H₂O (7:3). The residue obtained after evaporating the second eluate was transferred into a micro-tube with 0.5 ml of MeOH, and the solvent removed under an N₂ stream. The extract thus prepared was ready for reaction with DMEQ-TAD. To confirm the absence of interfering substances, control experiments were done on extracts prepared respectively from DG homogenates of nontoxic scallops and blue mussels. Recoveries of YTX during clean-up and derivatization procedures were tested by spiking the nontoxic scallop extracts with known amounts of YTX.

**Derivatization and clean-up.** To the extract placed in a micro-tube was added 50 µl of the DMEQ-TAD solution, and the reaction mixture was kept at room temperature in the dark for 2 h. Excess reagent was quenched with 50 µl of MeOH, and the reaction mixture was dried under an N₂ stream. The residue was loaded onto a Sep-pak column with 0.5 ml of MeOH/H₂O (3:7). After washing the column with 8 ml of MeOH/H₂O (3:7), the YTX adduct was eluted with 8 ml of MeOH/H₂O (7:3). The solution was evaporated under reduced pressure.

**HPLC conditions.** The HPLC system consisted of a Hitachi L-6000 pump, a Hitachi F-1050 fluorescence spectrophotometer, a Hitachi D-2500 chromato-Integrator (Tokyo), and a column oven (Gaskuro Kogyo Model 556, Tokyo). The separation column was a Cosmosil SC18AR column. For analysis of YTX and 45-OHTX a solution consisting of 40 mM phosphate buffer (pH 5.8) and MeOH (3:7) was used (mobile phase A). For determination of norYTX, a modified solution consisting of 35 mM phosphate buffer (pH 5.8) and MeOH (4:6) was used (mobile phase B). The flow rate was 1 ml/min, the column temperature was 35°C, and the excitation and emission wavelengths were 370 and 440 nm, respectively.

**Results and Discussion**

**Extraction and derivatization**

Approximately 90% of YTX was extracted in the supernatant. Repeated extraction of the residue resulted in only a 10% increase. Therefore, instead of repeating the extraction, we decided to do extraction only once and postulated that 1 ml of the supernatant contained 10% of the total YTX in the sample.

As predicted from the literature, DMEQ-TAD-YTX gave two peaks due to formation of (C-42) epimers in approximately 3:1 ratio (Fig. 3). Those peaks were isolated, and confirmed to show the same (M – Na)⁺ ion at m/z 1508 in the ESI-MS spectra. Tests with standard YTX indicated that the reaction was complete by 90 min. Taking the possible interference from sample matrices into account, the reaction time for shellfish samples was extended to 2 h. A good linearity between the peak areas and the YTX amounts used for derivatization was observed (Fig. 4). No interfering peak appeared in the chromatograms of nontoxic scallops and mussels. In triplicated experiments, the average recovery of spiked YTX was 94% in the range from 200 ng to 20 µg/g DG of scallops. Measurement of as little as 1 ng of YTX was possible. The sensitivity is 2000 times greater than the mouse bioassay, which requires 2 µg for detection.

**HPLC separation**

Figure 3 shows separation of the three adducts by mobile
phase A. The DMEQ-TAD adduct of norYTX was eluted close to interfering peaks. The separation was improved by changing the mobile phase to B. Because norYTX comprised only 2% of the total YTXs in scallops and has never been confirmed in other shellfish samples, routine analysis could be done with mobile phase A. The retention times of the YTXs were sensitive to fluctuation of the column temperature and the pH of the mobile phases. Hence, use of a column oven and calibration by standard toxins were imperative to obtain reproducible results.

Application to various samples

Occurrence of YTX and 45-OH-YTX in mussels from Chile and New Zealand was clearly shown for the first time (Fig. 5). After HPLC detection, YTX in these samples was isolated and confirmed by ESIMS. Detection of YTX in mussels from Oginohama is worth mentioning, because *Dinophysis* spp. were not detected in the sea when these mussels were collected. This supported the notion that *Dinophysis* were not responsible for YTX contamination. Significantly, 45-OH-YTX was more abundant than YTX in mussels, although the former comprised only 3% in scallops. Apparently, mussels actively biooxidize YTX to 45-OH-YTX. That phenomenon may have something to do with the more rapid depuration of toxicity in mussels than in scallops.

This HPLC method is superior to the current mouse bioassay method in rapidity, sensitivity, and specificity. Together with our previous method of measurement of DTX1 and OA, fluorometric HPLC methods provide information on precise toxin composition and thus are better alternatives to animal assays currently in use.

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