PSP Components of Toxicified Short-necked Clams in Yamagawa Bay, Kagoshima Prefecture

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Our efforts were made to clarify the cause of a food poisoning in which a person provoked numbness in the mouth by ingesting the short-necked clam *Tapes japonica* from Yamagawa Bay, Kagoshima Prefecture.

The toxic shellfish specimens which were collected from Yamagawa Bay were shucked and extracted with 80% ethanol acidified with acetic acid. After being defatted with chloroform, the aqueous extract was purified by chromatography on Bio-Gel P-2 and Bio-Rex 70. The toxins prepared were analyzed by cellulose acetate strip electrophoresis and thin-layer chromatography on silica gel. The results indicated that toxins contained protogonyautoxins (PXs) 1-4 as the major components, together with gonyautoxins (GTXs) 1-6 as the minor, on molar basis.

We thus came to a conclusion that the aforementioned poisoning was caused by the paralytic shellfish poisons (PSPs) accumulated in the short-necked clam.

On June 9-12, 1987, a large-scale red tide due to *Prorocentrum dentatum* occurred in Yamagawa Bay, Kagoshima Prefecture (Fig. 1). According to a survey of Kagoshima Prefectural Fisheries Experimental Station, the cell density at a depth of 0-5 m was highest in the inner bay (14,500-15,900 cells/ml) followed by the center (5,100-7,450 cells/ml) and the mouth (230-350 cells/ml) of the bay.

Although this red tide seemed not to exert harmful effects on wild and cultured fish, it caused infestation to bivalve molluscs such as short-necked clams presumably from the co-occurrence of *Protogonyaulax catenella*, a PSP producer with a highest cell density of 340 cells/ml.¹⁾

We collected short-necked clam specimens at the six stations established by Kagoshima Prefectural Government, Yamagawa Town Office and Yamagawa Fishermen's Cooperative Association, and examined the toxicity of shellfish using a mouse bioassay technique.

Since the toxicity values (5-6 MU/g) exceeding a quarantine limit of 4 MU/g were observed at stations B, C, D and E in the inner bay with the increased cell density of *P. catenella* (185-340 cells/ml), the Kagoshima Prefectural Government immediately promulgated a regulation against capturing and marketing of the shellfish in the bay area. However, the following day after the prohibition of marketing, a woman in the city of

Fig. 1. Surveillance of PSP in Yamagawa Bay, Kagoshima Prefecture. A-F are the stations established for monitoring the toxicity of short-necked clams, and 1-3 are those for phytoplankton survey.

Kagoshima was poisoned by ingestion of toxic short-necked clams which she had purchased from a Yamagawa peddler. The patient developed numbness of the mouth, but she was restored to

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Fig. 2. Various forms of PSP.

health in a half-day hospital care. We performed a mouse bioassay test on the suspected half-cooked shellfish which the patient left unfinished, and found that it carried a toxicity of 8 MU/g. The test animals evoked signs characteristic of paralytic shellfish poison (PSP).

In our laboratory toxins were separated from short-necked clams in the affected area to examine their chemical properties.

Materials and Methods

Materials

The short-necked clam *Tapes japonica*, which was toxified in the middle of June 1987 in Yamagawa Bay, was used for separation of PSP.

Purification Procedure

1. Extraction. The edible parts (1 kg, 0.55 MU/g) of the shellfish were homogenized for 3–5 min with two volumes of 80% ethanol adjusted to pH 3.7 with acetic acid. The homogenate was centrifuged at 3,000 rpm for 15 min. The resulting residue was treated three times with an equal volume of acidified 80% ethanol as above. The combined supernatant was concentrated *in vacuo* and shaken several times with a 1/3 volume of chloroform. The defatted aqueous layer was concentrated *in vacuo* and freeze-dried (27.6 g, 0.02 MU/mg).

2. Gel filtration on Bio-Gel P-2. The lyophilized sample (5 g, 100 MU) was dissolved in 15 ml of water, applied to a Bio-Gel P-2 (Bio-Rad Laboratory) column (2.2 × 50 cm), and eluted with 500 ml each of water and 0.15 M acetic acid. The water eluate was lyophilized and rechromatographed on the same column.

3. Chromatography on Bio-Rex 70. The toxic fraction (120 mg, 0.1 MU/mg) which was eluted first from the column was further purified on a

Bio-Rex 70 (acidic form, Bio-Rad Laboratory) column (1.4 × 50 cm) with a stepwise elution of acetic acid (0.05, 0.15 and 1 M). The toxic fractions obtained were lyophilized and analyzed as given below.

Toxin Standards

Standard toxins were separated by the methods reported previously: gonyautoxins (GTXs) 1–6 from the scallop *Patinopecton yessoensis*⁵²; proto-gonyautoxins (PXs) 1–4 from the oyster *Crassostrea gigas*⁵³; and saxitoxin (STX) from the crab *Zosimus aeneus*⁴³ (Fig. 2).

PX₁ and PX₂ correspond to Shimizu's GTX₈ and GTX₈ epimer,⁵³ or to Hall's C₁ and C₂⁴⁰; PX₃ and PX₄ to Hall's C₃ and C₄⁴⁷.

Methods of Analysis

1. Thin-layer chromatography (TLC). TLC was performed on a 5 × 20 cm silica gel precoated plate (Merck) with a solvent system of *tert*-butanol: acetic acid: water (2: 1: 1). Toxins were visualized as blue or yellowish blue spots under a UV lamp (365 nm) after being sprayed with 1% H₂O₂ and heated at 110°C for 5 min.

2. Electrophoresis. One microliter of the toxin solution (5–10 µg/µl) was applied to a 5 × 18 cm cellulose acetate strip (Chemetron) in 0.08 M Tris buffer (pH 8.7) and electrophoresed at 0.8 mA/cm for 15 min. For detection of the toxins the strip was treated as described in TLC.

3. High performance liquid chromatography (HPLC). To determine PSP compositions of the toxic fractions HPLC was carried out on a Hitachi 655 analyzer with a fluorescence monitor.⁴⁹ A stainless steel column (150 × 4 mm I.D.) with a water jacket (45°C) was packed with Hitachi 3013 C ion exchange resin and eluted with 0–0.15 M acetic acid.

4. Toxicity test. Toxicity was assayed by the

Fig. 3. Elution diagram of short-necked clam toxins from a Bio-Gel P-2 column.

official method for PSP.⁹⁾

Results

When the toxin extract (5 g, 20 MU/g) was applied to a Bio-Gel P-2 column, most of the toxicity was found in the water eluate (75 MU), but with a little toxicity also in the 0.15 M acetic acid eluate (5 MU). The water eluate, on rechromatography, was well separated into four toxic fractions (I-IV) (Fig. 3). The specific toxicities of I, II, III, and IV were 0.1, 4.1, 14.4, and 9.4 MU/mg, respectively.

On electrophoretic analysis (Fig. 4) relative mobility (R_m) of components of each fraction was compared with that of STX standard. Fraction I was resolved into four bands on the anode. Their R_m values corresponded to those of carbamoyl-*N*-sulfo-11 α -hydroxysaxitoxin sulfate (PX_1 , -0.20),³⁾ carbamoyl-*N*-sulfo-11 α -hydroxyne-*osaxitoxin* sulfate (PX_3 , -0.50)¹⁰⁾ and the respective 11 β epimers (PX_2 and PX_4 , -0.40 and -0.68). Fraction II or III formed two bands on the cathode (0.20 and 0.01) compatible with carbamoyl-*N*-sulfo-saxitoxin (GTX_6)¹¹⁾ and carbamoyl-*N*-sulfo-neosaxitoxin (GTX_8)¹²⁾ and four additional bands on the anode as seen in fraction I. Fraction IV included two bands each on both poles as in the preceding two fractions.

Similar results were also obtained in a comparison of R_f values on TLC. Fraction I was separated into four spots comparable to PX_1 (0.42), PX_2 (0.34), PX_3 (0.65) and PX_4 (0.45). Fraction II or

Fig. 4. Electrophoretic behaviours of the gel-treated short-necked clam toxins (fractions I-IV) on a cellulose acetate strip.

III contained six spots with R_f 's analogous to GTX_6 (0.26), GTX_8 (0.28), and the four compounds as noted in fraction I. The resolution of fraction IV resembled that of fraction III except that the

Fig. 5. Elution diagram of the gel-treated toxins (fraction I) from a Bio-Rex 70 column.

Fig. 6. HPLC analysis of the gel-treated short-necked clam toxins (fraction I).

former was deficient in two spots of PX_3 and PX_4 .

Fraction I was then further purified on a Bio-Rex 70 column. As shown in Fig. 5, Ia and Ib were eluted with water, and Ic, Id and Ie with 0.05 M acetic acid. Ia and Ib which appeared as small peaks on mouse toxicity accounted for more than 85% of the total yield on molar basis. Partial conversions of PX_{1-4} into GTX_{1-4} seemed to occur on treatment of fraction I with the Bio-Rex 70 column, although it remained to be studied on whether GTX_5 and GTX_6 would be the column artefacts or not. Anyhow, small quantities of GTX groups became detectable after the column treatment of fraction I. Ia was resolved electrophoretically into two bands with Rm and Rf

values corresponding to PX_3 and PX_4 ; Ib into PX_1 and PX_2 ; Ic into GTX_4 and GTX_6 ; Id into GTX_1 and GTX_3 ; and Ie into GTX_2 and GTX_5 .

The elution diagram of fraction I on HPLC appears in Fig. 6. I gave rise to eight peaks with retention times consistent with $PX_{1,2}$, $PX_{3,4}$ and GTX_{1-6} . The two major peaks due to $PX_{1,2}$ and $PX_{3,4}$ in this fluorescent spectrum were comparable to those of Ib and Ia as shown in the elution diagram of the preceding figure; GTX_{1-6} of higher toxicity appeared here as minor peaks.

Discussion

The toxicity of short-necked clams which became toxic in Yamagawa Bay in mid-June 1987 was monitored once a week until early July, and thereafter once a month until next March. The shellfish showed a toxicity of 6 MU/g in the inner bay on June 11, 1987, when the cell density at a depth of 0–1 m attained 86–185 cells/ml with *Protogonyaulax catenella* and 2,050–15,900 cells/ml with *Prorocentrum dentatum*. The shellfish toxicity decreased by 1–2 MU/g on June 23, 1987, 10 days after *Prorocentrum* and *Protogonyaulax* disappeared, and then down to less than 1 MU/g on June 30, 1987.

Toxicity of no more than 1 MU/g was detected from July 7, 1987, through January 28, 1988, but it again began to rise on February 23, 1988, by 1–2 MU/g in the appearance of *P. catenella*. This toxicity level was maintained from March through May 1988. On June 8, 1988, a red tide of *Heterosigma akashiwo* (9,600 cells/ml) occurred, but shellfish were not affected with this event.

In the Kagoshima region no intensive farming

of short-necked clams has been practised, though very popular in Kumamoto, a neighboring prefecture. Therefore, only wild shellfish have been captured here for marketing by the very limited number of fishermen. The clamming is open for public in most parts of this region. Under such circumstances Kagoshima Prefectural Government was of grave concern for surveillance of PSP. They stopped clamming and banned marketing of the shellfish, soon after the recognition of the toxicity surpassing the quarantine limit in the several stations of the bay. The degree of toxification, however, was relatively light, and exceeded no more than 8 MU/g in the shellfish examined.

In spite of the pertinent and urgent control measures of the Prefectural Government, the incidence of food poisoning by ingestion of toxic shellfish occurred in Kagoshima City. A patient (housewife, 41) ate the fried shellfish in butter (about 40 g) at supper. Within an hour after meal she complained of numbness around the mouth. As the symptom was persistent and seemed not to be improved soon, she was quickly hospitalized. On instillation overnight her health was restored completely.

Supposing this patient had ingested approximately 40 g of the shellfish with a toxicity of 8 MU/g, the manifestation of numbness might have ensued as low as 320 MU.

The above poisoning incidence has posed a very serious problem to the officials of Public Health Department of Kagoshima Prefecture on how to control PSP in wild marine organisms which are occasionally captured for marketing in the areas where the surveillance is unattainable. They are now asked to develop a good policy coping with this important problem. As a matter of course, the timely and proper measure of the administrative organ should be required at all times for prevention of human intoxication, but consumers as well as fishermen are also necessary to have a correct understanding of paralytic shellfish poisoning and to cooperate or follow the instructions of regional administrators by all means. The food poisoning case at this time largely owes

to the lack of cognizance of PSP.

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