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Changes in activity of hepatic xenobiotic-metabolizing enzymes of tiger puffer (*Takifugu rubripes*) exposed to paralytic shellfish poisoning toxins

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Abstract: *Attempts were made to examine the effect of paralytic shellfish poisoning toxins (PSP) on hepatic xenobiotic-metabolizing enzymes (XMEs) of tiger puffer (Takifugu rubripes). Two groups of nontoxic tiger fish were analyzed, and one group was fed with a PSP-containing diet (PSP group), and another with a PSP-free diet (control group). After 60 days of feeding, they were compared to each other, mainly in terms of the activity of XMEs. Both groups did not differ from each other significantly, in body weight gain, hepatosomatic index, and condition factor. Hepatic level of cytochrome P450 was lower in PSP group than control group. NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase, and ethoxyresorufin-O-deethylase (EROD) exhibited a reduced activity in PSP group than control group. Statistical analysis found that the activity or concentration of those enzymes correlated with the hepatic level of PSP, with r^2=0.497- 0.611.*

Key words: *Paralytic shellfish poisoning toxin (PSP), Puffer fish, Takifugu rubripes, Xenobiotic metabolizing enzyme (XME)*
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Introduction

Paralytic shellfish poisoning toxins (PSP) are one of the deleterious marine neurotoxins. It is produced mainly by planktonic dinoflagellate belonging to the genera *Alexandrium* and *Gymnodinium*, which are ingested by plankton feeders as represented by bivalves, leaving appreciable amounts of PSP in their digestive glands. Then, PSP may be transferred to upper trophic level organisms in the food chain. Recently, puffer was added to the list of PSP-bearing animals: Zaman *et al*. (1997) and Kungsuwan *et al*. (1997) successively found that freshwater puffer contained PSP exclusively, and indicated that this toxin could account for some serious poisoning cases in the tropics. Sato *et al*. (2000), on the other hand, disclosed that several species of marine puffer inhabiting the Philippines showed the presence of PSP in addition to another deleterious marine toxin, tetrodotoxin (TTX). Nakashima *et al*. (2004) also discovered that a Japanese marine puffer species contained comparable amounts of PSP and TTX, or PSP only, depending upon the organ.

Crabs of the family Xanthidae are also PSP-bearers. Noguchi *et al*. (1985) suggested that the crabs utilize this toxin as a defense agent positively. On the other hand, it is also suggested that many species of puffer use TTX for the same purpose (Saito *et al*., 1985a). By analogy, PSP-bearing puffer could accumulate and use this toxin as a defense substance. It is a pre-requisite for those puffers, to have a specific biological system by which they can accumulate PSP up to a high level. In fact, they showed much higher resistibility to PSP than other fish did, supporting for the presence of such specific system (Saito *et al*., 1985b), though the mechanism at molecular level remains to be elucidated as yet.

Living organisms are provided with a system consisting of many enzymes which decompose the xenobiotic substances and make it easier to excrete the resulting products: xenobiotic-metabolizing enzymes (XMEs). PSP could be regarded as a xenobiotic substance as well, and hence, we were much interested in its interaction with XMEs. In this connection, there have recently been published few related papers. Gubbins *et al*. (2000) gave the Atlantic salmon sublethal doses of PSP *via* i.p. route, finding that cytochrome P450 (P450) and glutathione-*S*-transferase (GST) were induced. It is assumed from this and other results that such induction could be provision for unfavorable changes of their living environment such as: infestation by red tides of PSP-producing plankton. On the other hand, Hong *et al*. (2003) administered orally PSP to several species of rodents, and found a reduced activity of XMEs such as pentoxyresorufin-*O*-depenthylase (PROD), ethoxyresorufin-*O*-deethylase (EROD), and superoxide dismutase (SOD). However, comparison of both these results is difficult, because of rather wide differences in experimental conditions.

In such a situation, we examined the effect of PSP on the activity of XMEs in the puffer (*Takifugu rubripes*). Young tiger puffers were fed with a PSP-containing diet for 60 days. After the feeding, livers were excised from them and assayed for the activity of XMEs.

Materials and Methods

PSP-containing diet: Crude PSP toxin was prepared from tropical crab *Zosimus aeneus* specimens which were collected from Ishigaki Island, Okinawa, Japan, as described previously (Arakawa *et al*., 1994). Briefly, their whole bodies were combined, minced, and extracted with 80% ethanol acidified with HCl (pH 2). After being defatted with dichloromethane, the extract was treated with activated charcoal, which was then eluted with 1% acetic acid in 20% ethanol to give a crude PSP solution. The toxin was composed essentially of saxitoxins (STXs) (Arakawa *et al*., 1995). A commercial fish meal and raw mackerel meat were mixed roughly at a 1:1 (w/w) ratio, to which the crude PSP solution was added at a level of 6.5 MU (mouse unit)/g. The mixture was thoroughly homogenized, and finally processed into a moist pellet form. Here, one MU is defined as the amount of PSP which kills a male mouse (body weight, 20 g) at 15 min after intraperitoneal injection, according to AOAC (Cunniff, 1995).

Feeding experiment: Seventy fish of 6-month-old, nontoxic tiger puffer [*Takifugu rubripes*; body weight, 67 ± 13 g; (Noguchi *et al*., 2004)] were fed with a PSP-containing diet for 60 days in a 1000 l aquarium which was of flow-through type, and well-aerated. The water temperature fluctuated from 15 to 24°C during the feeding. Their whole intake of the diet ranged from 40-110 g per day. Total dose of PSP per fish was calculated to be 790 MU on an average, or ca. 0.2 MU/day/g body weight. As control, another 70 fish of tiger puffer were fed in the same way, except with the PSP-free diet.

Seven specimens were randomly collected from each aquarium at the end of the feeding. Both these groups are referred to as PSP group and control group, respectively, for convenience. Hepatosomatic index (HSI, liver to body weight ratio), and condition factor (CF, body weight to cubic body length ratio) were measured as per standard methods (Lin *et al*., 2007).

Enzymic analysis: Livers were excised individually from those specimens, immediately frozen in liquid nitrogen, and kept frozen at –80°C prior to analysis. After thawing, the liver was rinsed with physiological saline, and homogenized in an ice-cold phosphate buffer (0.1 M KH_2PO_4/K_2HPO_4 and 20% glycerol, pH 7.4) with a Potter-Elvehjem type glass homogenizer. The homogenate was centrifuged (8,000 x g, 20 min., 4°C), and supernatant obtained was subjected to ultracentrifugation (100,000 x g, 120 min., 4°C). The resulting pellet was suspended in the above phosphate buffer, and used as microsomal fraction, whereas the supernatant as "cytosol fraction". Part of each liver was used to assay for PSP toxicity by the method described above.

XMEs assayed were P450 (Omura and Sato, 1964), P450R (Phillips and Langdon, 1962), b5R (Omura and Takesue, 1970), and three analogues of alkoxyresorufin-*O*-dealkylase (Burke and Mayer, 1974), which are EROD, methoxyresorufin-*O*-demethylase (MROD), and penthoxyresorufin-*O*-demethylase (PROD), on the microsomal fraction by the methods described in the respective references cited. Heme was determined by the method of Paul *et al*. (1953). GST was assayed on the cytosol fraction, according to Habig *et al*. (1974). Protein concentration was measured by the method of Lowry *et al.* (1951).

Statistics: Student t-test and regression analysis were done by SPSS (V. 10.0) and Microsoft Excel program, respectively, at 95% confidence intervals.

Results and Discussion

Effects of PSP on growth: After 60 days of feeding, average body weights of puffer from PSP group and control group were 102 ± 24 g and 95 ± 15 g, respectively, the difference being not significant ($p > 0.05$). Both groups did not differ significantly from each other in HSI either: 9.9 ± 0.9% for PSP group and 8.8 ± 2.3% for control group.

Effects of PSP on hepatic XMEs: Effects of PSP on hepatic XMEs from tiger puffer are collectively shown in Fig. 1. P450 concentration of PSP group was as low as 36% that of control group. PSP affected P450R and b5R as well, in essentially the same way. The ratio of activity (PSP group/control group) was 37% for the former enzyme, while 53% for the latter.

Among the three alkoxyresorufin-*O*-dealkylases, EROD exhibited a significantly lower activity in PSP group than control group, with the ratio of activity (PSP group/control group) of 39%. In contrast, MROD and PROD were not affected by PSP at all. GST was hardly affected by PSP either. As depicted in Fig. 2, heme concentration was somewhat lower in PSP group than control group, but the difference was not significant ($p > 0.05$).

Correlation between XMEs and hepatic level of PSP: The livers excised individually from PSP group exhibited toxicity scores, 3.0-8.3 MU/g liver, whereas those from control group showed scores below 3.0 MU/g liver without exception. The hepatic toxicity data of PSP group were plotted against the XME data, resulting in Fig. 3. The concentration of P450, and the activity of P450R, b5R and EROD were found to correlate with hepatic toxicity, with $r^2 = 0.497$-0.611 indicate significance level.

The main purpose of the present study was to clarify the effect of PSP on hepatic XMEs of tiger puffer.

To begin with, both groups did not differ significantly from each other in body weight gain, HSI, and CF. Hong *et al*. (2003) reported that mice administered with PSP showed a smaller body weight gain than control mice, though both mice were indistinguishable from each other in HSI.

As seen in Fig. 1, the concentration or activity of some XMEs differed between both groups of puffer. P450 concentration was decreased in PSP group. It may have been the biosynthesis of the apoprotein that was affected by PSP, because heme concentration was comparable between both groups, as described later. On the other hand, PSP reduced the activity of b5R and

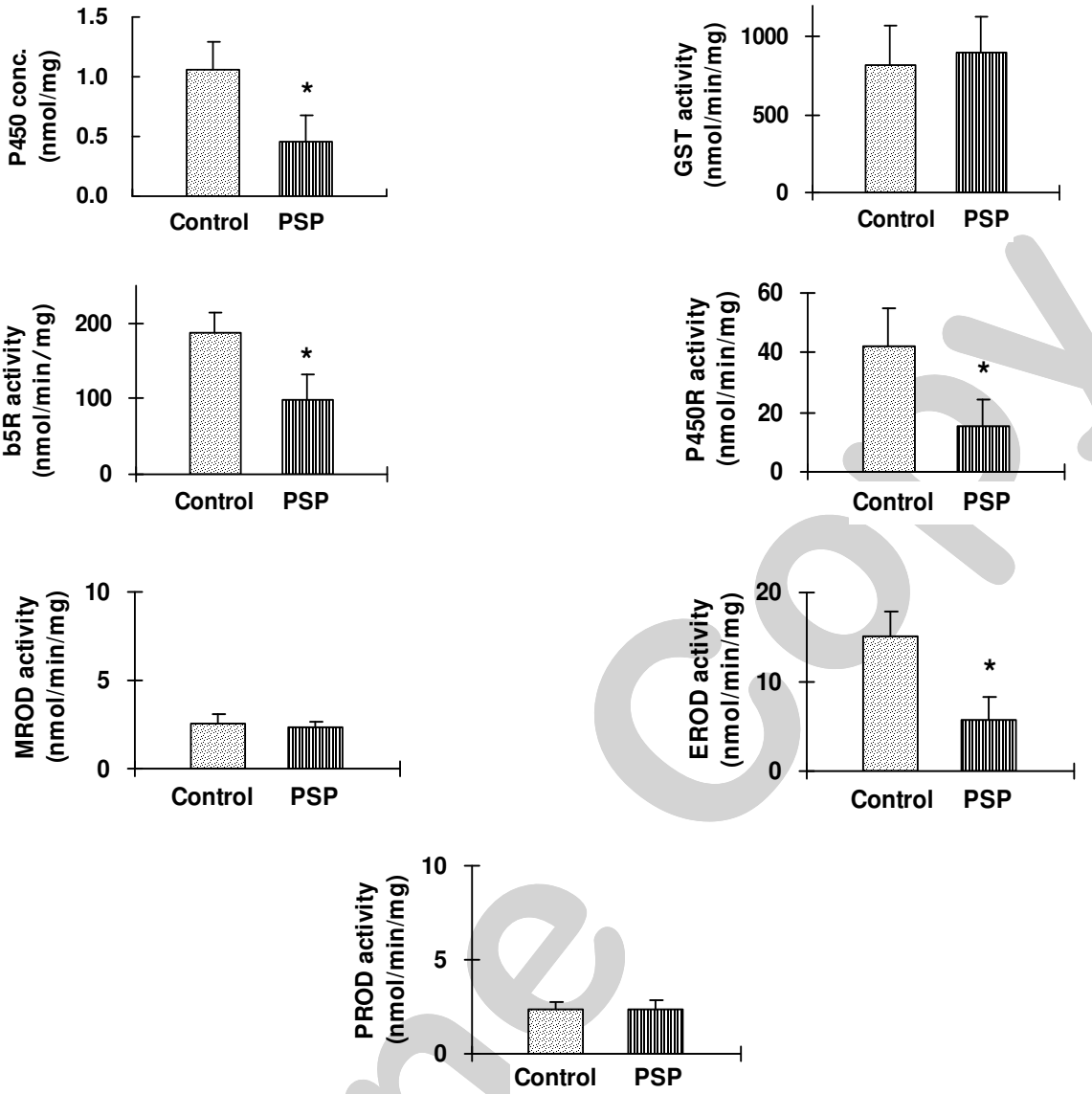

Fig. 1: Changes in concentration or activity of XMEs in tiger puffer liver, as caused by PSP. Data are shown by mean ± SD. Asterisk (*) represents the significant differences ($p < 0.05$) to control group

P450R. The effect was more pronounced in the latter enzyme. Among other XMEs, EROD showed a reduced activity in PSP group, compared to control group. MROD and PROD were not affected at all. In PSP-administered rodents, Hong *et al*. (2003) observed a reduced activity for EROD and also PROD. In the next place, our both groups did not show any significant differences in GST activity (Fig. 1). Gubbins *et al*. (2000) reported that PSP induced roughly double the activity of GST in Atlantic salmon.

As described above, the present results did not agree with those cited on some respects: *e.g*., body weight gain during feeding, the mode of effect of PSP on P450 concentration and GST activity. This may be attributed to not only species differences, but also to PSP dosage. In the case of GST, for example, our fish experimental was given a total of 790 MU/67 g body weight (B.W.), or by

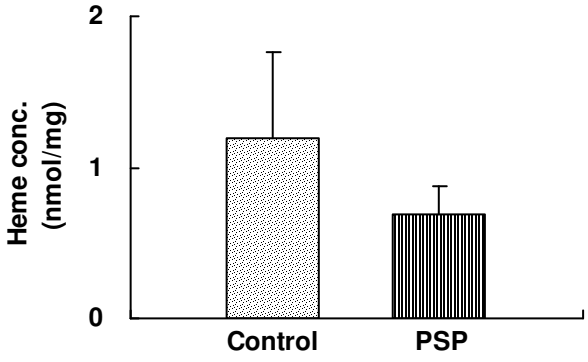

Fig. 2: Changes of heme concentration in hepatic microsome of tiger puffer, as caused by PSP. Data are shown by mean ± SD

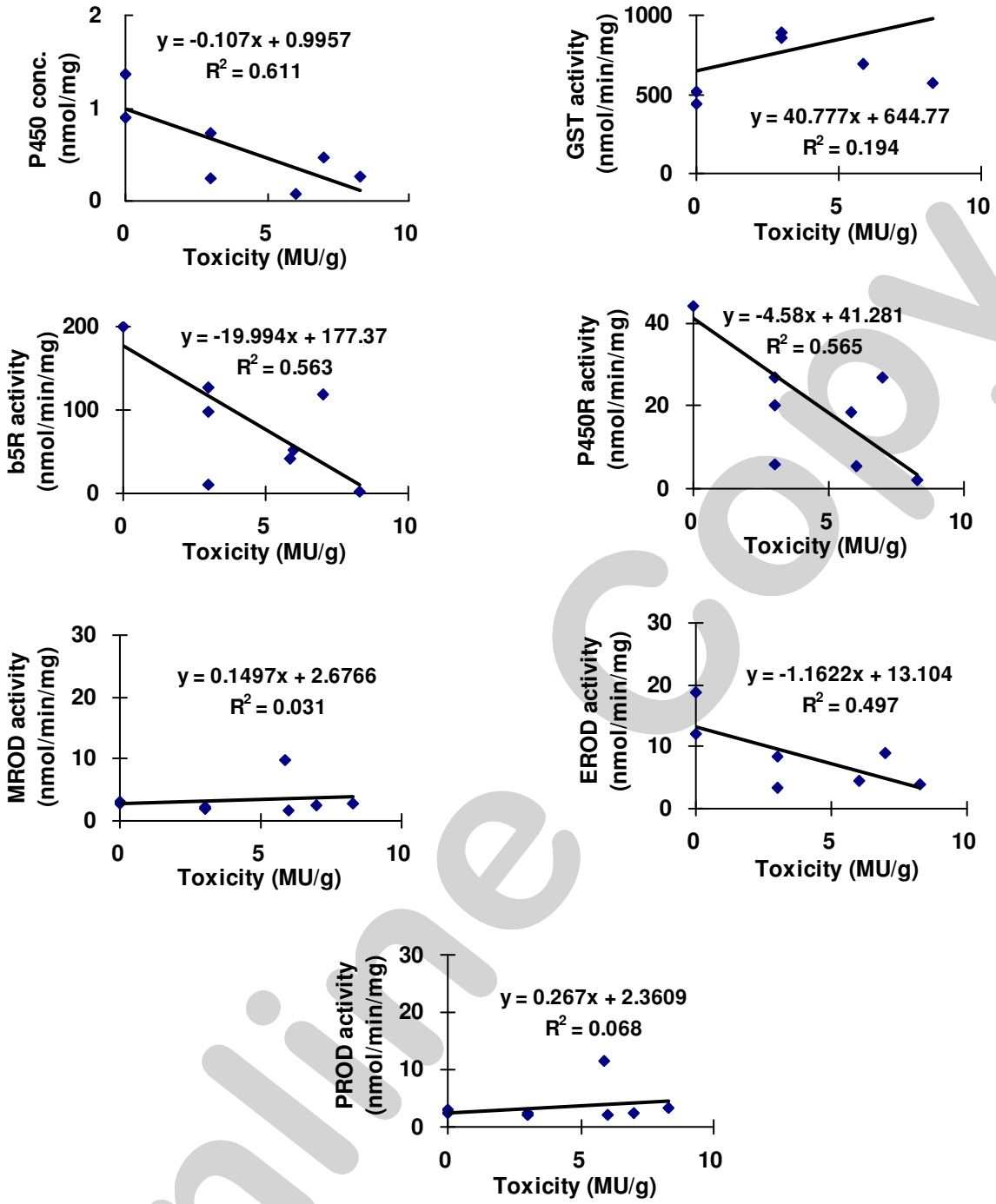

Fig. 3: Correlation (r^2) between PSP toxicity (MU/g) and the concentration or activity of XMEs in tiger puffer liver

conversion, 240 MU/20 g B.W. This dose is much larger than that in Atlantic salmon, 4.2 MU/20 g B.W. Incidentally, Hankinson (1995) observed the activity of scup P450 1A was induced by planar halogenated aromatic hydrocarbons at a low dose, whereas the same enzyme was inhibited by a high dose of an analogous substance (Gooch *et al*., 1989; White *et al*., 1997). Miranda *et al*. (1990) also reported the inhibitory action of another analogue in rainbow trout P450 1A.

As Fig. 3 shows collectively, the activity of b5R, P450R and EROD, along with P450 concentration, correlated with the hepatic PSP level, r^2 being 0.497-0.611. In contrast, the activity of MROD and GST did not correlate with the PSP level. It remains unexplained why the puffers reared under one and the same condition, accumulated PSP over such a wide range. Elucidation of PSP metabolism in fish is essential to reveal the reason.

Guengerich (2001) described that XMEs are implicated in the metabolism of hydrophobic substances such as steroids and fatty acids, with a resulting dysfunction. During the 60-day feeding, however, all puffers were in a good condition, as judged by appearances.

Further studies are now in progress, on the effect of PSP and other marine toxins on XMEs in fish.

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