Mechanism of the Reduction of Fenitrothion Toxicity in Kuruma Prawn by Piperonyl Butoxide

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A study on the relation between the toxicity of fenitrothion (FS) and its metabolism in kuruma prawn *Penaeus japonicus* treated with piperonyl butoxide (PB) was performed to elucidate the cause of the remarkable reduction of FS toxicity in the prawn by PB treatment, using ¹⁴C labeled FS.

The PB treatment did not affect FS absorption by the prawn from media, but it considerably inhibited the biotransformation of FS to fenitrooxon (FO), *i.e.* the reduction of FS toxicity in the prawn by PB treatment was attributed to the decrease in FO formation. The minimum lethal concentration of FO in the prawn was presumed to be 10-14 pmol/g, whereas that of FS was higher than 400 pmol/g. According to the amounts of FS and its metabolites found in kuruma prawn treated with and without PB, the main metabolic pathway of FS in the prawn was presumed to be FS→FO→desmethylfenitrooxon→3-methyl-4-nitrophenol→its conjugates.

In our previous paper,¹⁾ it was demonstrated that the toxicity of fenitrothion [*O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl)phosphorothioate; FS] in prawns was considerably reduced by treatment with piperonyl butoxide (PB) before and/or during exposure to FS. PB is well known as an effective inhibitor against the hepatic microsome monooxygenase.²⁾ Therefore, the reduction of FS toxicity in prawns by PB treatment may be attributed to inhibition of the biotransformation of FS to fenitrooxon [*O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl)phosphate; FO] which displays much higher toxicity than its parent compound.³⁾

The present study has been performed to elucidate the cause of the remarkable reduction of FS toxicity in kuruma prawn *Penaeus japonicus* by PB treatment, focusing on the relation between the effect of PB on FS metabolism and the reduction of FS toxicity.

Materials and Methods

Special Chemicals

Radioactive [ring-U-¹⁴C] fenitrothion (specific activity: 45 dpm/pmol; [¹⁴C] FS), non-radioactive

FS and its authentic metabolites such as desmethylfenitrothion (DMFS), FO, desmethylfenitrooxon (DMFO) and 3-methyl-4-nitrophenol (3-M-4-NP) were provided by Takarazuka Research Center, Sumitomo Chemical Co., Ltd. PB was purchased from Tokyo Kasei Kogyo Co., Ltd.

Enzymes

Three hydrolases were used for the determination of the conjugated metabolites of [¹⁴C] FS as follows:

- (1) β -Glucuronidase (EC 3. 2. 1. 31) (Sigma, Type IX from *Escherichia coli*)
- (2) β -Glucosidase (EC 3. 2. 1. 21) (P-L Biochem., from sweet almonds)
- (3) Arylsulfatase (EC 3. 1. 6. 1) (Sigma, Type H-1 from *Helix pomatia*)

Exposure of Prawn to [¹⁴C] Fenitrothion

Sixty kuruma prawns (average body weight, 2.8 g each) were divided into three groups (A, B, and C) as follows.

Group A: Ten prawns without PB pretreatment were placed in each of two glass aquaria containing 20 l of 5 ppb [¹⁴C] FS sea water (radio-

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activity, 810 dpm/ml; specific activity, 45 dpm/pmol; chlorinity, 17.3‰, as control.

Group B: Ten prawns without PB pretreatment were placed in each of two glass aquaria containing 20 l of 5 ppb [14 C] FS sea water supplemented with 0.25 ppm PB.

Group C: Ten prawns were pretreated with 0.25 ppm PB sea water for 12 h and then placed in each of two glass aquaria containing 20 l of the same [14 C] FS sea water as that for Group B.

PB and FS were dispersed into sea water with Tween 80 amounting to 10 and 20 times in weight, respectively. The experiment was carried out $25 \pm 1^\circ\text{C}$ with aeration. At appropriate intervals, four surviving prawns from each group were taken out, rinsed with FS-free sea water, weighed, frozen by dipping in liquid nitrogen, and then stored in a deep freezer at -80°C as a pooled sample, until being subjected to analysis for [14 C] FS and its metabolites accumulated in the prawns. Each dead prawn was taken out immediately after death and frozen by the above treatment. The dead prawns which had close survival times were pooled and stored at -80°C until being subjected to analysis.

Extraction of [14 C] Fenitrothion and Its Metabolites Accumulated in Prawn

The pooled prawns were homogenized with the same amount of distilled water by a Waring blender. The homogenates were subjected to the extraction of [14 C] FS and its metabolites with a benzene-ethanol mixture (9:1, v/v) and then with an ethyl ether-ethanol mixture (2:1, v/v), according to the procedure as described in our previous paper.⁴²

Determination of [14 C] Fenitrothion and Its Metabolites

1. Benzene- and ethyl ether-extracts: Appropriate aliquots of both extracts with benzene and ethyl ether were subjected to the measurement of radioactivity by a liquid scintillation counter (Aloka LSC-900; LSC), using an ACS-II scintillator (Amersham).

2. [14 C] FS and its benzene-extractable metabolites: [14 C] FS and benzene-extractable metabolites were separated by thin-layer chromatography (TLC) on silica gel plates (Merck 60 F₂₅₄) with carriers such as non-radioactive FS and its authentic metabolites, using two solvent systems: (I) toluene-ethylformate-formic acid (5:7:1, v/v) and (II) toluene-ethyl acetate-isopropanol-

acetic acid (8:12:5:3, v/v).

The R_f values of FS, 3-M-4-NP, FO, DMFS, and DMFO were 0.91, 0.76, 0.65, 0.22, and 0.11 in solvent system (I), and 0.95, 0.91, 0.83, 0.58, and 0.27 in system (II), respectively. The spots were detected under UV-light (254 nm), scraped off from the plates, put into glass vials with ACS-II scintillator, and then subjected to the measurement of radioactivity by LSC.

3. Ethylether-extractable [14 C] metabolites: As reported in our previous paper,⁴³ the [14 C] metabolites extracted with ethyl ether from the prawns after benzene-extraction were presumed to be some conjugates of [14 C] 3-M-4-NP. The conjugated [14 C] metabolites were subjected to enzymatic analysis, using suitable amounts of β -glucuronidase, β -glucosidase, and arylsulfatase, stepwise. The [14 C] hydrolyzate liberated from [14 C] conjugates by each enzyme was extracted with benzene and subjected to the measurement of radioactivity.

The amounts of [14 C] FS and its metabolites were calculated from their radioactivities on the basis of the specific activity of [14 C] FS (45 dpm/pmol) used in this experiment.

Results and Discussion

Effect of Piperonyl Butoxide on Fenitrothion Toxicity to Kuruma Prawn

As demonstrated in our previous paper,¹³ Fig.

Fig. 1. Effect of piperonyl butoxide (PB) on the toxicity of fenitrothion (FS) in kuruma prawn.

Fig. 2. Effect of piperonyl butoxide (PB) on the total amount of fenitrothion (FS) and its metabolites accumulated in kuruma prawn, during exposure to FS.

Four surviving prawns in each group at the respective exposure times were pooled and subjected to an analysis. The numbers of dead prawns pooled at the average survival times of 5.5, 8, 9, and 14 h were 4, 3, 2, and 2, respectively. One prawn in Group A died at 12-h.

1 shows a remarkable reduction in FS toxicity in kuruma prawn (Group C) treated with PB before and during FS exposure, resulting in no mortality during 24-h exposure to 5 ppb FS, while all prawns in Group A without PB treatment died within 12 h. The prawns of Group B treated with PB during FS exposure also showed an extended 50% survival time corresponding to approximately twice that in Group A.

Fenitrothion and Its Metabolites Found in Surviving and Dead Prawns

The loss of [14 C] FS in the test media at 24-h exposure was approximately 10% of the initial concentration. As shown in Fig. 2, the total amounts of [14 C] FS and its metabolites including conjugates found in surviving prawns increased as the exposure time was extended, but the amounts at the same exposure time did not show appreciable differences in the groups, demonstrating almost no effect of PB on FS absorption by the prawn. The prawns in Group C at 24-h exposure did not show any distress, in spite of their largest accumulation of [14 C] FS and its metabolites of all specimens including dead ones. The result indicates that the mortality of prawn during FS exposure is not due

to the total amount of FS and its metabolites.

Figure 3 shows the changes in the amounts of FS and FO accumulated in the prawns in each group. The amount of FS in prawns increased as the exposure time increased until 3 h, and then slowly. The FS content in Group C was higher than that in other groups, except the dead ones in Group A at 12-h, and the value reached 400 pmol/g at 24-h. The prawns in Group C, however, did not show any distress as mentioned above. The result suggests that FS is not a major mortal substance for kuruma prawn during FS exposure and that the lethal level of FS in the prawn is higher than 400 pmol/g.

On the other hand, the amount of FO accumulated in the prawns in Group A was extremely high of the three groups. The order of FO content in the groups was $A > B > C$, which was exactly the reverse of that in FS content, which was $A < B < C$.

The result demonstrates that the biotransformation of FS to FO in kuruma prawn was considerably inhibited by PB treatment, resulting in the low FO contents in PB-treated groups, especially Group C, mostly depending on the PB accumulated in prawns by PB pretreatment before FS exposure. In a further study the absorption

Fig. 3. Effect of piperonyl butoxide (PB) on the accumulation of fenitrothion (FS) and its oxon in kuruma prawn, during exposure to FS.

The experimental conditions, the prawn samples, and the expression of their appearance are the same as those in Fig. 2.

and excretion of PB in kuruma prawn will be elucidated. The higher FO contents found in the dead prawns compared with those in normal prawns indicate that FO is the major mortal substance and its minimum lethal concentration *in vivo* is approximately 10–14 pmol/g, as reported in our previous paper.³⁾

Although FO was detected in the prawns in Group A even at short FS-exposure times such as 1.5-h and 3-h, it was not detected at the same exposure time in the prawns in Group B, which had not been pretreated but were treated with PB during FS exposure. This means that the prawns abruptly absorbed PB from media at some level to prevent the oxidative desulfuration of FS absorbed simultaneously.

Although the total amounts of FS and its metabolites accumulated in the surviving prawns in the respective groups were on similar levels at the same exposure time (Fig. 2), the amounts of FS and its metabolites in the respective specimens greatly differed from group to group, as shown in Table 1.

The amount of FS in Group A ranged from 101

to 212 pmol/g between 1.5-h and 6-h exposure, while those in the PB-treated groups (B and C) ranged from 145 to 295 pmol/g and from 213 to 357 pmol/g, respectively. However, FO, DMFO, 3-M-4-NP, and conjugates except DMFO and conjugates in Group B at 6-h exposure showed almost the opposite trends to FS, whereas DMFS did not show any appreciable difference between groups, *i.e.* the formation of FS metabolites except for DMFS was inhibited by PB treatment. The lack of effect of PB on the demethylation of FS to DMFS is quite natural, because the reaction is catalyzed by glutathione S-transferase instead of monooxygenase.⁶⁾

According to the amounts of FS and its metabolites found in kuruma prawn treated with and without PB, the main metabolic pathway of FS in the prawn was presumed to be FS→FO→DMFO→3-M-4-NP→conjugates rather than FS→DMFS→3-M-4-NP→conjugates.

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Table 1. Effect of piperonyl butoxide (PB) on the amounts of fenitrothion (FS) and its metabolites accumulated in surviving kuruma prawns, during exposure to FS

Group	Exposure time (h)	FS	DMFS	FO	DMFO	3-M-4-NP	Conjugate	Other	Total
A	1.5	101.1 (52.2)	ND (0)	0.7 (0.4)	3.2 (1.7)	8.4 (4.3)	80.2 (41.4)	ND (0)	193.6 (100)
	3	212.6 (62.5)	1.1 (0.3)	2.2 (0.6)	5.5 (1.6)	18.9 (5.6)	99.8 (29.4)	ND (0)	340.1 (100)
	6	212.1 (43.1)	2.8 (0.6)	13.0 (2.6)	12.0 (2.4)	36.1 (7.3)	215.0 (43.7)	1.3 (0.3)	492.3 (100)
B	1.5	145.7 (79.1)	ND (0)	ND (0)	1.5 (0.8)	4.5 (2.5)	32.4 (17.6)	ND (0)	184.1 (100)
	3	241.4 (77.8)	0.9 (0.3)	ND (0)	2.5 (0.8)	9.5 (3.1)	56.0 (18.0)	ND (0)	310.3 (100)
	6	295.5 (50.7)	3.4 (0.6)	4.4 (0.8)	12.1 (2.0)	27.4 (4.7)	238.7 (40.9)	1.7 (0.3)	583.2 (100)
	12	345.5 (62.5)	10.6 (1.9)	7.9 (1.4)	8.6 (1.6)	13.2 (2.4)	165.3 (29.9)	1.7 (0.3)	552.8 (100)
C	1.5	213.4 (88.3)	ND (0)	ND (0)	0.9 (0.4)	4.4 (1.8)	23.0 (9.5)	ND (0)	241.7 (100)
	3	254.8 (82.2)	ND (0)	ND (0)	2.1 (0.7)	5.7 (1.8)	47.6 (15.3)	ND (0)	310.2 (100)
	6	357.4 (74.0)	3.9 (0.8)	1.6 (0.3)	5.4 (1.1)	11.1 (2.3)	102.9 (21.3)	1.0 (0.2)	483.3 (100)
	12	373.7 (54.4)	28.9 (4.2)	4.0 (0.6)	11.6 (1.7)	22.3 (3.2)	241.3 (35.2)	4.6 (0.7)	686.4 (100)
	24	414.6 (42.0)	16.8 (1.7)	7.8 (0.8)	26.3 (2.7)	45.1 (4.5)	475.2 (48.1)	2.1 (0.2)	987.9 (100)

The values are expressed as pmol/g-b.w. and (%) to the total radioactivity in the pooled four prawns at the respective exposure times.
 DMFS: desmethylfenitrothion; FO: fenitrooxon; DMFO: desmethylfenitrooxon; 3-M-4-NP: 3-methyl-4-nitrophenol; ND: not detected.

Group-A: Without PB-pretreatment and exposed to FS (5 ppb) without PB.

Group-B: Without PB-pretreatment and exposed to FS (5 ppb) with PB (0.25 ppm).

Group-C: With PB (0.25 ppm)-pretreatment for 12 h and exposed to FS (5 ppb) with PB (0.25 ppm).

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