# 有機リン剤抵抗性ニカメイガのフェニトロオクソン解毒因子としての薬物結合性タンパク


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**Short Communication**

# Binding Protein, a Factor of Fenitroxon Detoxication in OP-Resistant Rice Stem Borers*

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It has been widely known that insecticide metabolism by mixed-function oxidases, esterases and glutathione transferases is an important factor for insecticide resistance in insects. It has become clear that binding protein also plays an important role in diazoxon and paraoxon detoxication in an organophosphorus (OP)-resistant strain of green rice leafhopper, *Nephotettix cincticeps* UHLER.[1,2] At present, there is little information about binding protein related to insecticide resistance in other insects.

With respect to fenitrothion resistance in an OP-resistant strain of rice stem borer, *Chilo suppressalis* WALKER, which appeared in Okayama prefecture, we have found in *in vivo* studies that the increased detoxication of fenitroxon by cleavage of the P-O-aryl bond is the principal mechanism.[3,4] We recently developed a simple method to measure the activity of binding protein, and using the method we found that binding protein played an important role in fenitroxon detoxication in OP-resistant rice stem borers. This paper is to descrive the presence of binding protein and the detoxication mechanism of fenitroxon *in vitro* in OP-resistant rice stem borers.

The following Hata-f and S strains of rice stem borers were used in this study: Hata-f, an OP-resistant strain, was obtained from the OP-resistant Hata strain by selecting with fenitrothion for three successive generations. The original Hata strain was collected at Hata in Soja, Okayama prefecture in 1983, and maintained for four years without being exposed to insecticides. S, a susceptible strain, was collected at Kawashima-cho, Saitama prefecture in 1971 by the Institute of Physical and Chemical Research. The rearing methods of the two strains were described previously.[5] The $LD_{50}$ values of fenitrothion and fenitroxon topically applied to the Hata-f strain during this study were 69 and 27 $\mu$g/g, respectively, and those to the S strain were 1.8 and 1.2 $\mu$g/g, respectively. Fifth instar larvae (body weight: 60 to 70 mg) of the two strains were used.

Enzymes were prepared as follows. The whole bodies of 5th instar larvae were homogenized (20% w/v) with 1 mM EDTA–5 mM 2-mercaptoethanol–0.1 M phosphate buffer (EMP buffer), pH 7.4 in an ice bath. The homogenate was centrifuged at 600 g for 10 min to remove nuclei and debris. Mitochondria (10,000 g 10 min, ppt), microsomes(105,000 g, 60 min, ppt) and soluble fraction (105,000 g, 60 min, supernatant) were separated from the 600 g supernatant by centrifugation. The particulate fractions were suspended in EMP buffer.

*In vitro* metabolism of fenitroxon was examined by using [methoxy-$^{14}$C] fenitroxon (*O*, *O*-dimethyl *O*-3-methyl-4-nitrophenyl phosphate, 1.6 mCi/mmol, >99% purity) as a substrate. A reaction mixture consisting of 1 ml of enzyme solution and 10 $\mu$g of $^{14}$C-fenitroxon (in 10 $\mu$l EtOH) was incubated at 27°C. A mixture in which 1 ml of EMP buffer replacing the enzyme solution was the reference. Analytical procedures of the reaction mixture are shown in Fig. 1. After an appropriate time, 1 ml of 14% trichloroacetic acid (TCA) was added to the mixture to precipitate protein, and then 3 ml of $CHCl_3$ was added to remove unchanged fenitroxon. Radiocarbon of the water-soluble fraction resulted possibly from enzymatic hydrolysis and of the $CHCl_3$-soluble fraction was measured by LSC with a dioxane scintillator (dioxane: naphthalene: PPO=950: 45: 5). TLC procedures to identify metabolites were described previously.[4] The protein precipitate was filtered off, washed with TCA and $CHCl_3$ and solubilized with 1 ml of NCS (purchased from Amersham), and radiocarbon bound to protein was measured by LSC with a toluene scintillator (toluene: POPOP=997: 3).

Aliesterase (nonspecific esterase) activity to $\beta$-naphthyl acetate (NA) was assayed according to the method of Motoyama *et al.* (1984).[1]

Time course protein binding and hydrolysis of fenitroxon in the two strains of rice stem borers

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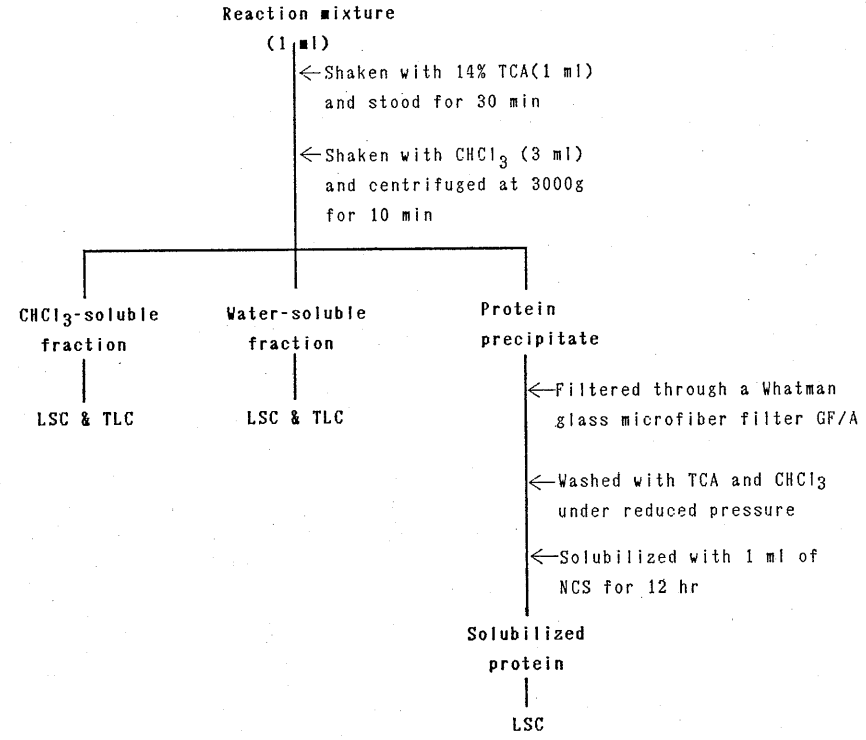


Fig. 1 Diagram showing procedures to analyze the detoxication activity of fenitroxon.

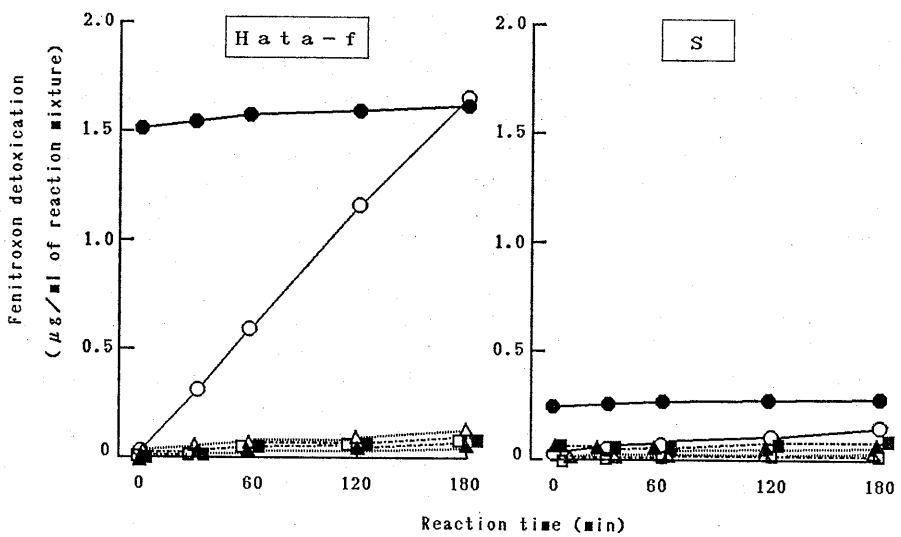


Fig. 2 Time-course protein binding and hydrolysis of fenitroxon after incubation with each fraction from the Hata-f and S strains of rice stem borers.

Fenitroxon binding (protein precipitate) by the soluble fraction (●), microsomes (■), and mitochondria (▲); fenitroxon hydrolysis (Water-soluble fraction) by the soluble fraction (○), microsomes (□), and mitochondria (△).

are shown in Fig. 2. In the Hata-f strain, a considerable amount of radiocarbon was detected in both the water-soluble fraction and the protein precipitate from the soluble fraction. Particularly, radiocarbon recovered from the protein precipitate almost reached an equilibrium state at zero-time incubation, and the equilibrium state continued up to 180 min. No radiocarbon was found in the protein precipitate when the soluble fraction was boiled at 100°C for 1 min. The radioactivity in the precipitate was detected only in the protein fraction by gel filtration, dialysis and ammonium sulfate precipitation. The elution peak coincided completely in position with those of proteins and $^{14}$C-fenitroxon when the mixture of $^{14}$C-fentiroxon and the soluble fraction from the Hata-f strain was chromatographed in a Sephadex G-25 gel-filtration system. These findings indicate that the radiocarbon detected in the protein precipitate is of binding protein, and suggest that protein binding is an important mechanism for fenitroxon detoxication in the Hata-f strain. In addition, the radiocarbon in the water-soluble fraction increased with time, suggesting that hydrolysis also plays an important role in detoxication in this strain. A metabolite in the water-soluble fraction was identified as dimethyl phosphate by cochromatography with authentic standard.

The optimum pH for the two detoxication systems in the Hata-f strain was examined (buffers used: 0.1 M phosphate, pH 5.3–8.0; 0.1 M tris, pH 7.6–9.0). The optimum pH for the hydrolysis of fenitroxon was 7.4, and the protein-binding activity was constant between pH 5.3 and 9.0.

We have demonstrated that the salioxon-analog K-2 is an effective synergist for fenitrothion and fenitroxon against the OP-resistant rice stem borers,[3,5] and that the degradation of fenitroxon *in vivo* is strongly inhibited by K-2.[3] Effect of K-2 on the two detoxication systems in the Hata-f strain was examined: K-2 inhibited strongly both detoxication activities. The $I_{50}$ values of K-2 against binding protein and hydrolysis were $1.3\times10^{-5}$ M and $7.1\times10^{-6}$ M, respectively.

In the S strain, these two fenitroxon detoxication activities were markedly low (Fig. 2), *i.e.*, the binding activity was 7 times lower and the hydrolytic activity 9 to 10 times lower than the Hata-f strain. The experimental evidence obtained from the metabolism of fenitrothion and fenitroxon *in vivo*,[3,4] synergism[3,5] and the present study, have led us the conclusion that the increased activity of the two detoxication systems is responsible for the fenitrothion resistance in the Hata-f strain.

Table 1 Aliesterase activity to β-naphthyl acetate of the Hata-f and S strains of rice stem borers.[a]

| Strain | Aliesterase activity | |
|---|---|---|
| | μmol/min/larva | μmol/min/mg protein[b] |
| Hata-f | 11 | 2.7 |
| S | 12 | 2.8 |

a) The soluble fraction was used as an enzyme source.

b) Protein contents of the soluble fractions from the Hata-f and S strains were 4.3 and 4.4 mg/larva, respectively.

It has been revealed that OP-resistant green rice leafhoppers have higher aliesterase activity to β-NA than the susceptible strain,[2,6-8] and that aliesterase has a dual role of catalyst for hydrolysis of malathion and fenvalerate and of binding protein for paraoxon in the resistance mechanism.[1] In the case of rice stem borer, no quantitative and qualitative differences were observed in aliesterase between the Hata-f and S strains, *i.e.*, the aliesterase activity to β-NA was almost the same in the two strains (Table 1), and there was no difference in the aliesterase sensitivity to fenitroxon between the Hata-f ($I_{50}$ value, $6.3\times10^{-9}$ M) and S ($6.3\times10^{-9}$ M) strains. The results suggested that aliesterase is not responsible for the binding protein for fenitroxon in the Hata-f strain.

Presence of binding protein in OP-resistant green rice leafhoppers is shown by measuring free *p*-nitrophenol liberated from paraoxon[1] and by an AChE inhibition test with diazoxon,[2] but not by direct measurement of oxon-bound protein. This study is the first study in which binding of $^{14}$C-fenitroxon to protein was directly measured. In order to clarify the binding mode of fenitroxon to protein, *i.e.*, whether covalent or noncovalent, further *in vitro* study is now underway.

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## 要　　約

### 有機リン剤抵抗性ニカメイガのフェニトロオクソン解毒因子としての薬物結合性タンパク

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有機リン剤抵抗性ニカメイガにおけるフェニトロオクソン解毒機構について *in vitro* で検討した．有機リン剤抵抗性(Hata-f)および感受性(S)系統の各5齢幼虫より調製した酵素液と［メトキシ-$^{14}$C］フェニトロオクソンとの反応から，加水分解により生成したジメチルリン酸が水層に見いだされた．また，水，クロロホルムに不溶で，TCAで沈殿する区分に$^{14}$Cが検出され，ゲル濾過，熱変性等からこれはフェニトロオクソンと結合しているbinding proteinであることがわかった．加水分解およびタンパクとの結合はともに可溶性画分で著しく高かった．Binding proteinはpH依存性の加水分解と異なり，pH 5.3～9.0の範囲内でフェニトロオクソンと急速に結合し，またアリエステラーゼとは異なることが示唆された．Binding活性と加水分解活性は，Hata-f系統がS系統にくらべてそれぞれ7倍，9～10倍高いことから，この解毒活性の差がHata-f系統のフェニトロチオン抵抗性の主要因と考えられる．