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Mechanisms of Parathion Resistance in a Ethyl Fenitrothion-Selected Yumenoshima III Strain of House Flies

페니트로치온 도태 Yumenoshima 저항성 집파리에 있어서의 파라치온 저항성 메카니즘

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ABSTRACT The biochemical factors responsible for parathion resistance in a ethyl fenitrothion-selected Yumenoshima III (EF-30) strain of the housefly were examined. Great difference (167-fold) in the I_{50} was observed between the resistant EF-30 (R) and susceptible SRS (S) strains *in vitro*, suggesting that altered acetylcholinesterase (AChE) in the housefly strain was an important factor in the resistance. The *in vitro* degradative activity of parathion and paraoxon in both strains was associated with the microsomal and soluble fractions and required NADPH and reduced glutahione (GSH), respectively. The R strain possessed higher activity for GSH *S*-transferase than the S strain, and this enzyme appears to be important in the resistance mechanism. The R strain was highly resistant to parathion (101,487-fold), but substitution of the methoxy group for ethoxy group decreased the resistance level (25,914-fold) and parathion could be a substrate of GSH *S*-transferase. It is concluded that the combination of some factors (altered AChE, and enhanced activity of cytochrome P_{450} dependent monooxygenase and GSH *S*-transferase) could be sufficient to account for the extremely high level of resistance to parathion and parathion-methyl, although a possible involvement of other factor(s) can not be excluded.

KEY WORDS Housefly, parathion, resistance mechanism, biochemical factor

초 록 Yumenoshima III 집파리 계통을 ethyl fenitrothion으로 30세대 도태시킨 EF-30 계통에 있어서의 parathion 저항성 메카니즘을 생화학적으로 조사하였다. 아세틸콜린에스테라제 저해활성은 저항성계통과 감수성 SRS 계통간에 커다란 차이를 보여 이 효소의 감수성 저하가 저항성의 주요 메카니즘으로 작용하고 있음을 알 수 있었다. 양 계통에 있어서의 parathion과 paraoxon의 *in vitro* 분해활성은 미크로좀 및 수용성 분획과 관련이 있으며, 각각 NADPH와 glutathione을 필요로 하였다. 저항성계통은 감수성계통에 비하여 GSH *S*-transferase 활성이 높아 이 효소가 저항성 메카니즘에 중요한 역할을 하고 있는 것으로 추정되었다. 저항성계통은 parathion에 대하여 101,487배, ethyl parathion에 대하여 25,914배의 저항성비를 나타내어 parathion이 GSH *S*-transferase의 기질로 작용하고 있음을 알 수 있었다. 이상의 결과로부터 EF-30 계통에 있어서의 저항성 메카니즘에는 수종의 요인이 관여하여 parathion에 대하여 높은 저항성을 나타냄을 알 수 있었으나, 이들 요인 이외에 타 요인의 관여를 배제 할 수 없었다.

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The history of resistance to insecticides in the Danish housefly shows that the appearance and development of resistance are an inevitable and stepwise biological process (Keiding 1977). Organophosphorus insecticides (OPs) have been used for control of houseflies in garbage dumps or dairy farms in Japan since the late 1960s. The intensive use caused decrease in susceptibility to OPs or other groups of insecticides. Yasutomi (1975) reported the significant development of OP resistance in the housefly which was collected from a man-made garbage dump in Tokyo Bay in 1974. The strain, referred to as Yumenoshima III strain,

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has shown high levels of resistance to various kinds of insecticides until 1986 (Kudamatsu 1983, Yasutomi *et al.* 1988).

Resistance mechanisms of houseflies to OPs are known to be reduced penetration, insensitivity of target sites, and enhanced detoxifying enzyme activity. However, a reduced penetration mechanism has been identified as a *pen* factor on chromosome III of houseflies which alone gives no resistance or enhanced resistance slightly (2- to 5-fold) and acted as an intensifier of other resistance factors (Plapp & Hoyer 1968). The biochemical aspects of resistance to OPs in some Japanese housefly strains have been studied (Motoyama *et al.* 1980, Ugaki *et al.* 1985, Shono 1986, Lee 1996). The factors involved in the resistance are mainly enhanced metabolism by mixed function oxidases (MFO) and GSH *S*-transferases, and reduced sensitivity of AChE. Esterases including phosphatases have been also reported to be an important factor for OP resistance in some Japanese strains of the housefly (Shono 1974a, Motoyama *et al.* 1980, Ugaki *et al.* 1985).

In the laboratory study described herein, we investigated biochemical mechanisms of resistance to parathion by subcellular fractions of the susceptible SRS and the ethyl fenitrothion-selected EF-30 strains of housefly.

MATERIALS AND METHODS

Houseflies

Two housefly strains were used. SRS (S), the WHO standard reference susceptible strain, was supplied by Universita di Pavia, Italy and has been reared without exposure to any insecticide in the laboratory. An EF-30 strain (R) had been produced by selecting an OP resistant Yumenoshima III strain with ethyl fenitrothion for 30 generations at a selection pressure of 30 to 60% mortality for succesive generations. Selection was performed on adult flies <10 hr old by topical application method. Flies which survived 24 hr after treatment were maintained at 25°C, 65±2% relative humidity (RH), and a photoperiod of 16: 8 (L: D) hr to produce offspring.

Chemicals

Parathion, parathion-methyl, paraoxon, and paraoxon-methyl were obtained from Sumitomo Chmical, Ltd, Osaka, Japan. These compounds were either of an analytical grade. [Ring-2,6-^{14}C] Parathion and [ring-2,6-^{14}C] paraoxon were purchased from Amersham, Buckinghamshire, England and had a specific activity of 21 mCi/mmol and 11 mCi/mmol, respecticely. DTNB [5,5-dithiobis (2-nitrobenzoic acid)], DCNB (1, 2-dichloro-4-nitobenzene) and eserine salicylate were purchased from Tokyo Kasei Chemical Company, Tokyo, Japan. All other chemicals used were of reagent grade.

Toxicity Test

Toxicity to parathion and its related compounds was determined by applying 0.5 μl of solutions of the insecticides in acetone topically to the thorax of 4- to 6-day-old female adult flies. Treated flies were held at 25°C, 65±2% RH and a photoperiod of 16: 8 (L: D) hr, and mortalities were determimed 24 hr after treatment. Data from all bioassays were corrected for control mortality using Abbott's (1925) formula. LD_{50} values and slopes were estimated by probit analysis (Raymond 1985). A resistance ratio (RR) was calculated according to the formula RR = LD_{50} value of the R strain/d by LD_{50} value of the S strain.

Enzyme Assay

The abdomens of 4-to 6-day-old female flies (150 abdomens/15ml) were homogenized in 0.2 M phosphate buffer (pH 8.0). The homogenate was filtered through cheese cloth. The nuclear plus debris (650 g× 10 min precipitate), mitochondrial (10,000 g×15 min precipitate), microsomal (210,000 g×20 min precipitate) and soluble (210,000 g×20 min supernatant) fractions were obtained by differential centrifugation as previously described (Brattsten & Gunderson 1981) using a Beckman L-78 ultracentrifuge. The precipitate was washed twice with the same buffer. In order to protect the enzymes from endogenous inhibitors, 1%

bovine serum albumin was included in the buffer throughout the preparations (Motoyama *et al.* 1980).

GSH *S*-transferase activity was assayed spectrophotometically by the method of Booth *et al.* (1961). The reaction mixture contained 0.1 ml of the supernatant, 3.4 ml of 0.1 M Tris-HCl buffer (pH 9.0) and 0.4 ml of 40 mM DCNB in ethanol. The reaction was run at 30°C and the change on absorbance was recorded at 344 nm using a Cary Model 14 recording spectrophotometer. All reactions were corrected for nonenzymatic hydrolysis using boiled enzyme preparations.

In Vitro Degradation of Parathion and Paraoxon

The reaction mixture contained 0.5 ml of the enzyme solution, 10.5 nmol [^{14}C] parathion and 10.5 nmol [^{14}C] paraoxon and either 5 mM NADPH or 10 mM GSH in a total volume of 2 ml. After incubation at 30°C for 30 min, the mixture was partitioned with 2 ml chloroform and the amoumt of radioactivity in the water layer was determined with a Packard Tri-Carb scintillation counter.

AChE Inhibition

The *in vitro* inhibition of AChE by paraoxon and paraoxon-methyl was measured by the method of Ellman *et al.* (1961), using acethylthiocholine iodide as the substrate. Flies kept at -20°C were decapitated by shaking and heads homogenized in ice-cold 0.1 M phosphate buffer (pH 8.0). After filtering through cheese cloth, the homogenate was centrifuged at 500 g for 5 min. The supernatant was used directly as the AChE preparation. Incubation mixture consisted of 0.5 ml of enzyme solution (equivalent to 0.5 female heads), 2.5 ml of 0.1 M phosphate buffer, 0.2 ml of 3 mM DTNB in buffer and 10 μl of various concentrations of paraoxon and paraoxon-methyl in ethanol. The reaction mixture was incubated at 30°C for 10 min and 0.2 ml of 32 mM acethylcholine iodide was then added to the mixture. After incubation for 20 min at 30°C, the reaction was stopped by adding 0.2 ml of 5 mM eserine salicylate. The AChE activity was measured at 412 nm.

RESULTS

The Yumenoshima III strain after 14 generations of selection by ethyl fenitrothion showed cross-resistance to diazinon (LD_{50}, 190 μg/fly), parathion (LD_{50}, 64 μg/fly), fenitrothion (LD_{50}, 500 μg/fly), fenitrothion-ethyl (LD_{50}, 300 μg/fly), malathion (LD_{50}, >500 μg/fly) and chlorpyrifos-methyl (LD_{50}, >500 μg/fly). After 30 generations of selection, the toxicity to parathion and related analog to the EF-30 and SRS strains of houseflies was topically determimed (Table 1). The R strain was highly resistant to parathion (101,487-fold), but substitution of the methoxy group for the ethoxy group decreased moderately the resistance level (25,914-fold). This strain also was rasistant to paraoxon and paraoxon-methyl, but to a lesser extent than parathion and parathion-methyl.

The *in vitro* inhibition of AChE from the R and S strains of houseflies by various concentrations of paraoxon and paraoxon-metyl was examined to ascertain their contribution to the resistance mechanism (Table 2). AChE from the R strain exhibited a marked

Table 1. Toxicity of parathion and related compounds to resistant EF-30 and susceptible SRS strains of houseflies

Compound	EF-30[a]	SRS	RR[b]
	LD_{50}, μg/fly (95% CL)	LD_{50}, μg/fly (95% CL)	
Parathion	395.8 (377.8~414.8)	0.0039 (0.0037~0.0041)	101,487
Parathion-methyl	513.1 (474.2~569.7)	0.0198 (0.0194~0.0202)	25,914
Paraoxon	218.7 (191.7~251.4)	0.0646 (0.0586~0.0709)	3,385
Paraoxon-methyl	102.2 (98.5~106.2)	0.0562 (0.0521~0.0622)	1,819

[a]This strain had been produced by selecting an OP resistant Yumenoshima III strain with ethyl fenitrothion for 30 generations at a selection pressure of 30 to 60% mortality.

[b]Resistance ratio = LD_{50} value of R strain/LD_{50} value of S strain.

decrease in sensitivity to inhibition by two OPs than that from the S strains. Insensitivity ratios (I_{50} value of the R strain/I_{50} value of the S strain) against paraoxon and paraoxon-methyl were 167 and 169, respectively.

The GSH *S*-transferase activity in both strains was determined using DCNB as the substrate. As shown in Fig. 1, it was obvious that the R strain had higher activity for the substrate than the S strain.

The *in vitro* degradation of [ring 2,6-^{14}C] parathion by subcellular fractions from the R and S strains was investigated in the presence and absence of cofactors (Table 3). The data showed that degradation of parathion was associated with the soluble fraction and the reactant required GSH as cofactor.

The addition of GSH to the soluble fraction resulted in a 10- and 2-fold increase in water soluble metabolites by the R and S strains, respectively. The R strain exhibited more than a 8-fold higher activity in the system than the S strain. However, microsomal degradation was not dependent on NADPH.

The *in vitro* degradation of [ring 2,6-^{14}C] paraoxon by subcellular fractions was also investigated (Table 4). It was observed that degradation by the microsomal fraction increased upon the addition of NADPH and the R strain exhibited higher activity than the S strain. Similary, degradation by the soluble fraction was enhanced by the addition of GSH and again the R strain showed much higher activity than the S strain.

Table 2. Inhibition of fly-head acetylcholinesterase from resistant EF-30 and susceptible SRS strains of houseflies by paraoxon and paraoxon-methyl

Inhibitor	I_{50} (M)		Ratio[a]
	EF-30	SRS	
Paraoxon	1.4×10^{-7}	8.4×10^{-9}	167
Paraoxon-methyl	7.6×10^{-6}	4.5×10^{-8}	169

[a]I_{50} value of R strain/I_{50} value of S strain.

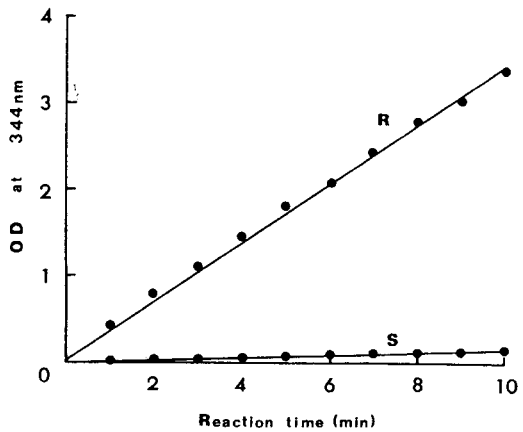

Fig. 1. Glutathione *S*-transferase activity in the soluble fractions from resistant EF-30 (R) and susceptible SRS (S) strains of houseflies. DCNB was used as substrate.

Table 3. Degradation of [ring 2,6-^{14}C] parathion by subcellular fractions of housefly abdomen homogenates from resistant EF-30 and susceptible SRS strains of houseflies[a]

Fraction	Cofactor	Water-soluble metabolites[b]	
		EF-30	SRS
Nuclei	None	0.27±0.03	0.27±0.03
Mitochondria	None	0.43±0.03	0.37±0.03
	NADPH	0.43±0.03	0.37±0.03
	GSH	0.47±0.03	0.43±0.03
Microsome	None	0.57±0.03	0.57±0.03
	NADPH	1.17±0.07	0.60±0.06
Soluble	None	0.93±0.03	0.73±0.09
	GSH	9.30±0.21	1.17±0.13

[a]Data were obtained from three replicate experiments. Total recovery of radioactivity in each experiment was >90%.
[b]Percentage of applied radioactivity recovered as water-soluble metabolites of parathion.

Table 4. Degradation of [ring 2,6-^{14}C] paraoxon by subcellular fractions of housefly abdomen homogenates from resistant EF-30 and susceptible SRS strains of houseflies[a]

Fraction	Cofactor	Water-soluble metabolites[b]	
		EF-30	SRS
Nuclei	None	0.20±0.00	0.17±0.03
Mitochondria	None	0.20±0.06	0.10±0.00
	NADPH	0.37±0.03	0.12±0.04
	GSH	0.10±0.00	0.09±0.01
Microsome	None	0.30±0.06	0.33±0.03
	NADPH	3.78±0.22	0.50±0.00
Soluble	None	0.40±0.06	0.27±0.03
	GSH	2.50±0.12	0.43±0.03

[a]Data were obtained from three replicate experiments. Total recovery of radioactivity in each experiment was >90%.
[b]Percentage of applied radioactivity recovered as water-soluble metabolites of paraoxon.

DISCUSSION

The world-wide famous OP resistant Yumenoshima III strain was selected with ethyl fenitrothion for 30 generations. Its cross resistance and the extremely high levels of resistance to parathion (101,487-fold) and parathion-methyl (25,914) suggest that multiple factors might be involved in the resistance.

There was a remarkable interstrain difference in the sensitivity of AChE to the inhibition by paraoxon and paraoxon-methyl *in vitro*. The reduced sensitivity is caused by structurally altered AChE. There are at least two different types of AChE (Tripathi & O'Brien 1973, Devonshire & Moores 1984). The altered AChE has been verified in a number of Japanese strains of houseflies as a common mechanism of OP resistance (Motoyama *et al.* 1980, Yeoh *et al.* 1981, Ugaki *et al.* 1983, Lee 1996), although this mechanism was first found in *Tetranychus urticae* Koch (Smissaert 1964).

The importance of the MFO in the insecticide metabolism and its role in resistance are well recognized (Hodgson & Kulkarni 1983, Wilkinson 1983, Hodgson 1985). In our study, microsomal degradation was dependent upon NADPH, suggesting that MFO might play some role in parathion resistance mechanism in the housefly, although with this R strain an increase in degradation was also found in the soluble fraction, These results indicate that another factor(s) may be involved in the resistance.

The significance of GSH *S*-transferases in the metabolism of OPs and their resistance mechanism has been established (Shono 1974b, Motoyama & Dauterman 1980, Motoyama *et al.* 1980, Ugaki *et al.* 1985). This enzyme is known to have a preference of methoxy group of the substrates and shows poor activity toward ethoxy, propoxy, or butoxy groups (Motoyama & Dauterman 1972, 1975). In our study, the R strain of housefly degraded more parathion and paraoxon into water soluble metabolites in the soluble fraction than did the S strain *in vitro*. The greatest difference in degradation was observed when GSH was added as a cofactor. Therefore, it is probable that the higher degradation of parathion by the R strain is due to GSH *S*-transferase and increased activity of this enzyme in the R strain is at least in part, responsible for the mechanism of resistance to parathion in the housefly. Motoyama & Dauterman (1972, 1975) reported that the resistance factor to *O,O*-dimethyl-substituted azinphosmethyl had a good correlation with this degradation by GSH *S*-transferase as a resistance mechanism. The highest resistance and enzyme activity were observed with *O,O*-dimethyl analogs and the resistance and the enzyme activity decreased greatly when the methyl group was substituted by ethyl, propyl, or butyl group. However, our results showed that the R strain was highly resistant to parathion (105,130-fold), but substitution of the methoxy group for ethoxy group decreased the resistance level (26,000-fold), and parathion could be a substrate of GSH *S*-transferase.

The present study demonstrated that more than one biochemical and physiological mechanism was involved in parathion resistance in the ethyl fenitrothion-selected Yumenoshima III strain of housefly. The combination of some factors (altered AChE, and enhanced activity of MFO and GSH *S*-transferase) would be sufficient to account for the extremely high level of resistance to parathion, although involvement of other factor(s) such as phosphatases can not be excluded.

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