Activity and Substrate Specificity of the Esterase Associated with
Organophosphorus Insecticide Resistance in the Kanzawa
Spider Mite, *Tetranychus kanzawai* KISHIDA
(Acarina : Tetranychidae)¹

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Organophosphorus insecticide resistance in the Kanzawa spider mite is associated with an increase in esterase activity to naphthyl acetate, tributyrin, phenyl acetate and methyl-*n*-butyrate. All of these esters except phenyl acetate are hydrolyzed by esterase (aliesterase) which is resistant to eserine inhibition and heat labile in slightly alkaline media, whereas an appreciable part of phenyl acetate hydrolysis is due to cholinesterase which is sensitive to eserine inhibition. A good correlation between aliesterase activity (naphthyl acetate hydrolyzing activity) and co-toxicity coefficient of a mixture of malathion and K-1 in organophosphorus insecticide resistant strains was recognized. Tributyrin acts as a competitive inhibitor for β -naphthyl acetate hydrolysis and phenyl acetate acts as a noncompetitive inhibitor. No inhibition of β -naphthyl acetate hydrolysis was observed when methyl-*n*-butyrate was added.

INTRODUCTION

Two distinct opposite observations on the relationship between organophosphorus insecticide resistance and nonspecific esterase activity have been reported in the spider mite, i.d. resistance associated with high esterase activity and resistance associated with low esterase activity toward naphthyl acetate hydrolysis. The former phenom-

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enon was reported in the citrus red mite (MOTOYAMA, 1968; TAKAHASHI et al., 1971) and the latter in the two-spotted spider mite (SMISSAERT, 1965; MATSUMURA and VOSS, 1964).

Organophosphorus insecticide resistant strains of the Kanzawa spider mite demonstrated increased activity in degrading malathion *in vitro* when compared to the susceptible strains. Electrophoresis revealed the presence of esterase bands which exhibited not only elevated β -naphthyl acetate hydrolyzing activity but also malathion hydrolyzing activity (KUWAHARA et al., 1981).

A relationship between organophosphorus insecticide resistance and nonspecific esterase activity in the Kanzawa spider mite has not been reported. Therefore, as a preliminary step toward the characterization of esterases involved in organophosphorus insecticide resistance in this mite, the substrate specificity was studied and additional analyses of the enzymes hydrolyzing some esters were made.

MATERIALS AND METHODS

Mite strains. Four organophosphorus insecticide-resistant strains and one reference susceptible strain of the Kanzawa spider mite were used for these studies. The NsD, NsP and NsM strains were established by selecting the Ns strain with dichlorvos, phenthoate and malathion, respectively. Selection pressure was maintained by varying the dose so that about 30% of the female mites survived to propagate the succeeding generation over a 10-generation period. The origin and response to chemicals of the Ns strain has been described elsewhere (KUWAHARA, 1977). The Zo strain originated in a resistant field population in Shimizu, Shizuoka prefecture, where it was found on strawberry grown in a greenhouse. The strain was originally heterogeneous in susceptibility to organophosphorus insecticides but females were selected four times with dichlorvos at 30 ppm to eliminate the susceptible individuals. The reference strain NsPs was established by reversing selection of the Ns strain with diazinon at 10 ppm for 10 generations as described elsewhere (KUWAHARA, 1977). According to the dosage-mortality curve, the resistant mites and the reference mite did not include any susceptible or resistant individuals, respectively.

Pesticides and synergist. The following acaricides and synergist were used for the study. Dichlorvos was obtained from Nippon Soda Co., Ltd.; malathion from Sumitomo Chemical Co., Ltd.; phenthoate from Nissan Chemical Ind. Ltd.; K-1, (2-phenyl-4H-1,3,2-benzodioxaphosphorin 2-oxide), a specific synergist of malathion, was synthesized as described elsewhere (ETO and OSHIMA, 1962). Those compounds with more than 99% purity were formulated as 50% (W/V) E. C. (50 : 20 : 10 : 5 : 10 : 5; AI : acetone : dimethylformamide : monochlorobenzene : xylene : Sorpol® 9838 emulsifier).

Substrate. α - and β -naphthyl acetate(NA), tributyrin(TB), phenyl acetate(PA) and methyl-*n*-butyrate(MB) were obtained commercially. These esters were dissolved in ethanol containing 2% Triton X-100.

Measurements of esterase activity. Esterase activity of a single homogenized female toward α - and β -naphthyl acetate was measured as described by VAN ASPEREN (1962) and that of mite homogenates toward tributyrin, phenyl acetate and methyl-*n*-butyrate were measured according to the method of HESTERIN (1949). A single female mite was crushed with a spatula in 0.01 ml of glass-distilled water on a glass slide until no

tissue debris was observed. The resultant brei was rinsed into a test tube with 1 ml of phosphate buffer (0.067 M, pH 7.0). The mites were collected by brushing them from bean leaves and were used regardless of their sex and developmental stage. Homogenates were prepared in an ice-cold glass homogenizer. The reaction mixture consisted of 0.01 ml of substrate with a final concentration of 5×10^{-4} M of NA and 1×10^{-3} M of TB, PA and MB, and 1 ml of mite homogenate. The substrate concentrations used were sufficient to ensure maximum rates of hydrolysis in each case. After incubation at 30°C for 20 min, 0.5 ml of diazoblue lauryl sulfate solution (DBLS) was added. The DBLS was prepared by mixing 2 parts of a 1% solution of diazoblue B with 5 parts of a 5% solution of sodium lauryl sulfate. Esterase activities were measured at 550 nm for β -NA, at 600 nm for α -NA and at 540 nm for TB, PA and MB. The amount of esters hydrolyzed was proportional to the homogenate concentrations and the reaction period up to 60 min. In studies on the kinetics of esterase inhibition *in vitro* with K-1, 0.01 ml of inhibitor dissolved in ethanol containing 2% Triton X-100 was added 10 min prior to the addition of the substrate and the residual esterase activities in homogenates of the NsPs and NsM strains were determined. The bimolecular rate constants (k_2) were determined according to the method of ALDRIDGE (1950).

Acaricide treatment. Female mites were sprayed with aqueous suspensions of acaricides via a rotary type spray tower. To analyze the synergism of malathion with K-1, female mites were treated with a mixture of malathion and K-1 (0.1%). A single application of K-1 did not show any acaricidal effect at the concentrations applied. Twenty-four hr LC50 values were determined graphically as previously described (KUWAHARA, 1977). Results are expressed as resistance factors, i.e., ratios of LC50 of each strain to that of the reference susceptible strain, the NsPs. The co-toxicity coefficient of a mixture was obtained by the method of SUN and JOHNSON (1960).

RESULTS

The LC50 values of malathion and dichlorvos against the reference strain NsPs and the resistance factors for the other strains of female mite are shown in Table 1. The LC50 and the calculated resistant factors were not significantly different in resistance among the selected strains. It is of interest that the selection procedure employed in this study resulted in nearly similar levels of resistance to malathion and dichlorvos in all the strains selected.

Esterase activities to α - and β -NA of a single homogenized female and those to TB, PA and MB of the mite are shown in Tables 2 and 3, respectively. All the esters were hydrolyzed at appreciable rates and some difference in esterase activity between

Table 1. Response of NsPs strain to malathion and dichlorvos and resistance factors in resistant strains

Pesticide	LC50 NsPs strain (ppm)	Resistance factor			
		NsD	NsM	NsP	Zo
Dichlorvos	19.1	35.6	28.7	27.2	32.3
Malathion	4.2	2590	3904	3290	3013

Table 2. Esterase activity toward naphthyl acetate in five strains of mite

Strain	Esterase activity $\pm$ S.D. ^c		Activity ratio (a/b)
	α -NA ^a	β -NA ^b	
NsPs	4.31 $\pm$ 0.09	4.08 $\pm$ 0.08	1.06
NsD	5.02 $\pm$ 0.16 (1.17)	4.61 $\pm$ 0.14 (1.12)	1.09
NsP	6.71 $\pm$ 0.31 (1.56)	6.43 $\pm$ 0.29 (1.56)	1.04
NsM	7.80 $\pm$ 0.26 (1.81)	7.09 $\pm$ 0.33 (1.10)	1.10
Zo	6.59 $\pm$ 0.35 (1.53)	5.91 $\pm$ 0.30 (1.44)	1.12

^c S. D. (standard deviation). Data were average of sixty individual females.

Activity was expressed as 1×10^{-5} M substrate hydrolyzed/female/20 min. Figures in parentheses show relative activity in resistant strains to reference strain NsPs.

Table 3. Esterase activities toward phenyl acetate, tributyrin and methyl-*n*-butyrate in homogenates of five strains of mite

Substrate	Strain				
	NsPs	NsD	NsP	NsM	Zo
Phenyl acetate	5.57	5.99	6.37	7.14	6.20
Tributyrin	1.19	1.52	1.93	2.09	1.89
Methyl- <i>n</i> -butyrate	2.69	2.88	3.46	4.00	3.01

Enzyme concentration: 1.43 mg mite/ml phosphate buffer. Figures are average of 10 replications and are expressed as 1×10^{-4} M substrate hydrolyzed/mg mite/20 min.

Fig. 1. Relationship between esterase activity to α - (●) and β -naphthyl acetate (○) and co-toxicity coefficient of a mixture of malathion and K-1. Esterase activity was expressed as 1×10^{-5} M naphthyl acetate hydrolyzed/female/20 min.

the strains was observed. The OP-resistant strains, especially the malathion selected strain (NsM), manifested increased esterase activity as compared with that of the susceptible strain (NsPs). There was no correlation between esterase activity and resistance to acaricides in the resistant strains. The increased activities observed in these strains were less than twice that of the NsPs.

A good relationship between NA-hydrolyzing esterase activity and synergism of a mixture of malathion and K-1 is shown in Fig. 1. It is obvious from the data

that the more α - and β -NA esterase activity found in the mite, the higher is the co-toxicity coefficient of the mixture of malathion and K-1. The increased esterase activities involved in resistant strains, therefore, are concerned with the detoxification of malathion, as a single application of K-1 did not exhibit any acaricidal effect at the concentrations tested.

The rate of β -NA hydrolysis in the homogenate of the susceptible NsPs and the resistant NsM strains in the presence of different concentrations of eserine and acetylcholine is shown in Table 4. Extremely high concentrations of eserine and acetylcholine were ineffectual in the inhibition of β -NA hydrolysis. Only about 30% inhibition of the mite homogenates was obtained with 1×10^{-2} M eserine. The results obtained in this study suggest that a small portion of the β -NA hydrolysis (less than 3%) was due to the cholinesterase (ChE), as the ChE activity of both strains was completely inhibited by 1×10^{-6} M eserine (KUWAHARA, unpublished data) and it is known to inhibit only ChE (and not other esterases) at a concentration up to 1×10^{-6} M (SMISSAERT, 1965).

The effect of pH and heating to 50°C for 10 min and 60 min on esterase activity to β -NA and MB from the NsPs strain is shown in Fig. 2. Esterase activity was found to increase considerably from pH 5.59 to pH 8.04. The activities to β -NA and MB at pH 5.59 were about 37.7% and 69% of those at pH 8.04, respectively. Heating

Table 4. Effects of eserine and acetylcholine on esterase hydrolyzing β -naphthyl acetate

Strain	Eserine			Acetylcholine	
	1×10^{-6} M	1×10^{-4} M	1×10^{-2} M	5×10^{-5} M	5×10^{-2} M
NsPs	97.8	88.9	67.5	100	100
NsM	98.6	93.3	74.6	100	100

Enzyme concentration: 0.0143 mg/ml phosphate buffer. Figures show percentage of normal activity remaining after inhibition. Inhibitors were added to homogenates 10 min prior to addition of substrate.

Fig. 2. Influence of pH and temperature on esterase activity of NsPs strain. Figures in parentheses are time for denaturation. Enzyme concentration: 0.0143 mg mite in 1 ml phosphate buffer (0.067 M, pH 7.0) for β -naphthyl acetate hydrolysis and 1.43 mg mite in 1 ml phosphate buffer for methyl-*n*-butyrate hydrolysis. ●—● : β -naphthyl acetate; ○—○ : methyl-*n*-butyrate.

to 50°C for 10 min and 60 min showed that part of the esterase activity was heat labile in slightly alkaline media, but relatively stable in acidic media. The activity to β -NA was reduced to 59% and 24% of normal after 10 and 60 min, respectively. The activity to MB was completely inactivated by heating to 50°C for 10 min in alkaline media, but the inactivation rate was much lower when the homogenate was prepared in acidic media.

These results prompted a further study on the effect of temperature on β -NA activity in homogenates of the NsPs and NsM strains under slightly alkaline conditions (pH 8.04). The systems were heated to 38°, 44° and 50°C for 5 to 120 min and the residual activities were determined at 25°C (Fig. 3). Storing the homogenates for 120 min at 25°C caused less than 2% loss of activity. An appreciable difference in sensitivity to heat inactivation was found between the strains. Esterase activity of the NsM was found to decrease to about 40% of the normal activity at 50°C for 5 min, whereas that of the NsPs was about 70% of normal. Thus, part of the esterase activity of the OP-resistant strain was more sensitive to heat inactivation than that of the susceptible strain. Thereafter, a slight decrease in β -NA hydrolysis occurred in the two strains, even when the heat inactivation was continued for 120 min. Approximately 25% of the activity was still active. Thus, an enzyme(s) which is resistant to heat inactivation and which is present in equal amounts in OP-resistant and -susceptible strains was involved in part of the hydrolysis of β -NA. The results obtained in this study clearly demonstrate that this eserine-resistant and heat-alkali-labile enzyme which hydrolyzes β -NA is an aliesterase (AliE) (METCALF et al., 1956).

The effect of eserine and K-1 on esterase activity to PA, TB and MB is shown in Table 5. The esterase activities in the homogenates of the NsPs strain to TB and

Fig. 3. Heat denaturation of NsPs and NsM esterase at pH 8.04.

○—○ : NsM; ●—● : NsPs

Table 5. Inhibition of esterase activity in homogenates of NsPs strain by eserine and K-1

Substrate	Eserine		K-1	
	5×10^{-8} M	5×10^{-6} M	5×10^{-8} M	5×10^{-6} M
Phenyl acetate	81	69	76	26
Tributyrin	100	100	26	0
Methyl- <i>n</i> -butyrate	100	100	0	0

Enzyme concentration: 1.43 mg mites/ml phosphate buffer. Figures show percentage of normal activity remaining after inhibition. Inhibitors were added to the homogenates 10 min prior to addition of substrate.

Fig. 4. *In vitro* inhibition of esterase hydrolyzing β -naphthyl acetate in homogenates of NsPs and NsM strains by K-1.

MB were insensitive to eserine, but very sensitive to K-1. On the other hand, part of the hydrolysis of PA was inhibited by low concentrations of eserine and was less sensitive to K-1. Thus, TB and MB are hydrolyzed by an AliE but PA is hydrolyzed by both a ChE and an AliE, and the latter enzyme is, at least quantitatively, the more important.

Figure 4 shows the effect of the concentration of K-1 on the percentage of initial esterase activity of homogenate to β -NA. The effect of K-1 concentration on the esterase activity in homogenates of the NsPs and NsM strains showed essentially first order reaction rates for the irreversible inhibition of esterase activity in homogenates from both strains and the involvement of at least three enzymes with bimolecular rate constants (k_2) in the order of 10^2 (I), 10^3 (II) and more than 10^3 mole $^{-1}$ min $^{-1}$, respectively. Enzymes I and II of both strains have almost the same constants and an equal amount of homogenate activity. Thus, an enzyme(s) which is rather insensitive to K-1 and which is present in an approximately equal amount in the OP-susceptible and -resistant strains is responsible for the hydrolysis of β -NA. By extrapolation of the lines to zero time for the NsPs and NsM enzyme the ordinates were intercepted at 70% activity for the former and at 50% for the latter, indicating a possible heterogeneity of the esterase and 20% of the enzyme being present in a more sensitive form in the NsM than in the NsPs.

The results obtained in this study indicated that OP-resistance was associated with increased esterase activity to NA, TB, PA and MB, and some differences in sensitivity to eserine, K-1 and heat inactivation were demonstrated. These results prompted a further analysis of the esterase(s) hydrolyzing these esters and competition of NA with TB, PA and MB for AliE was examined. Figure 5 shows the Dixon plots of β -NA hydrolysis involving variable concentrations of inhibitors. When β -NA was the substrate, TB acted as a competitive inhibitor which showed an intersection above the abscissa (Fig. 5, A), whereas PA acted as a noncompetitive inhibitor which showed an intersection on the abscissa (Fig. 5, B). Therefore, TB shares the same active site as β -NA on the enzyme, whereas PA has a different active site from β -NA. On the other hand, no inhibition of β -NA hydrolysis took place when MB was added to the homogenate (Fig. 5, C). Thus, the esterase(s) hydrolyzing MB is quite different from that hydrolyzing β -NA. The inhibition constants (k_i) of TB and PA for β -NA hydrolysis obtained by the method of LINEWEAVER and BURK (1934) were 2.44 ± 0.13 mM and 11.52 ± 0.38 mM, respectively. Thus, these esters are good inhibitors for β -NA hydrolysis.

Fig. 5. Dixon plot of β -naphthyl acetate hydrolysis to show competitive inhibition by tributyrin (A), noncompetitive inhibition by phenyl acetate (B) and no inhibition by methyl-*n*-butyrate (C). Concentration shown is that of substrate.

DISCUSSION

There have been many reports which demonstrated a correlation between OP-resistance and increased esterase activity in insects (KASAI and OGITA, 1965; OZAKI and KASAI, 1965; STONE and BRAWN, 1969; YASUTOMI, 1970; NEEDHAM and SAWICKI, 1971; HAMA and IWATA, 1972) and predaceous mites (MOTOYAMA, 1971). In some cases, the esterase activities of the resistant strain were more than ten times that of the susceptible strain. A similar phenomenon has been reported with spider mite (MOTOYAMA, 1968; TAKAHASHI et al., 1972), however, the difference in esterase activity between them was relatively small when β -NA was used as substrate. The results obtained in this study are in full agreement with these findings (Tables 2 and 3).

From the data given in Table 2, no significant difference was found between the esterase activity in OP-susceptible and -resistant strains, if α - or β -NA was used as the substrate. On the other hand, the AliE activity to α -NA of OP-susceptible strains of housefly was more than 6 times higher than that of the OP-resistant strains, whereas no significant difference in the esterase activities to β -NA between susceptible and resistant strains of houseflies was found (VAN ASPEREN, 1962). Thus, mite AliE has almost the same activity to these substrates, which in turn means that this AliE has a relatively low substrate specificity for the two naphthyl acetates, and is quite different from that of the housefly.

There was no correlation between interstrain difference in esterase activity and resistance to acaricides (Tables 1 and 2). A very good correlation has generally been

obtained between *in vivo* resistance and the *in vitro* insensitivity of AChE to inhibitors except for malathion in these strains (KUWAHARA, unpublished data). This would indicate that the insensitivity of AChE to OP-inhibitor is the major mechanism of resistance in the mite as has been demonstrated previously (SMISSAERT, 1964; Voss and MATSUMURA, 1965; ZAHAVI and TAHORI, 1970). In the case of malathion, however, there was a good correlation between esterase activity and synergism of a mixture of malathion and K-1. Agar gel electrophoresis of mite homogenates from the OP-resistant strains demonstrated the presence of esterase bands which showed not only increased activity to β -NA but also increased malathion hydrolyzing activity. These bands were selectively inhibited by K-1 (KUWAHARA et al., 1981). Considering the results, there is little doubt that increased enzyme activity toward β -NA in the resistant strains is, at least in part, directly related to the detoxification of malathion, whereas the increased AliE activity toward MB in resistant strains is not related to the metabolism of malathion. MB did not compete with β -NA for the AliE (Fig. 5), indicating the possibility of two or even more enzymes being present with slightly different substrate specificity.

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