

Two Types of Carboxyesterase Degrading Malathion in Resistant Houseflies and their Inhibition by Synergists. Hideo OIKAWA, Morifusa ETO, Yasuyoshi OSHIMA†, Fumikazu TANAKA* and Kanehiro UMEHA* (Department of Agricultural Chemistry, Kyushu University, Fukuoka and *Institute of Agricultural Chemicals, Toa Noyaku Co. Ltd., Odawara) Received August 26, 1968, *Botyu-Kagaku* 33, 139, 1968.

19. 抵抗性イエバエに存在する2種類のマラチオン分解カルボキシエステラーゼとそれらのマラチオン共力剤による阻害 大川秀郎, 江藤守穂, 大島康義, 田中文一*, 梅田兼弘* (九州大学農学部農芸化学科, *東亜農薬株式会社農薬研究所) 43. 8. 26 受理

サリゲニン環状リン酸エステルのフェニル誘導体 (k-2) はイエバエ, ツマグロヨコバイに対して malathion と共力効果を示した。特に, malathion 抵抗性系で高い共力効果が認められた。malathion 抵抗性系イエバエ, ツマグロヨコバイのエステラーゼは寒天ゲル電気泳動法によって分離された。それらのエステラーゼのうち, malathion と共力効果を示す有機リン剤, EPNO, *n*-propyl paraoxon, k-2 で阻害されるものは carbophenoxy malathion あるいは malathion の carboxy-ester を分解した。また, それらのエステラーゼ活性は malathion 抵抗性系で感受性系よりも高かった。malathion 抵抗性系のイエバエには malathion を分解する少くとも2種類のエステラーゼが存在することが電気泳動的に明らかとなった。その中で, 陰極側に移動するものは malathion の分解により大きく関与していた。しかし, 陽極側に移動する酵素は弱い malathion 分解力しか示さなかった。k-2 の malathion 共力効果は malathion を分解するカルボキシエステラーゼの阻害によるものと考えられる。

Introduction

Saligenin cyclic phosphorus esters analogous to active metabolite of tri-*o*-cresyl phosphate (TOCP) have been examined on biological activities^{1,2}. Small alkyl derivatives are highly insecticidal,³ whereas aryl derivatives are selective inhibitors of ali-esterases and appear to be synergistic with malathion.⁴ The synergism is more prominent in resistant houseflies than in a susceptible strain.⁵ The specificity of saligenin cyclic phosphorus esters in the housefly esterase inhibition was also revealed by means of agar gel electrophoresis. When the exo-cyclic substituent was changed, saligenin cyclic phosphorus esters exhibited characteristic antiesterase activity.⁶

Malathion being low toxic to mammals may be detoxified by the enzymatic hydrolysis either of the phosphate ester bond or of the carboxy-ester bond. It has been observed that the latter enzyme activity is much higher in malathion resistant strains of mosquitoes (*Culex tarsalis*)⁷, houseflies (*Musca domestica*)⁸ L.) and green rice

leafhopper (*Nephotettix cincticeps* Uhler)⁹ than in susceptible strains. Attempts were made to characterize the carboxyesterase responsible for resistance to malathion by Matsumura and Hogendijk⁹. On the other hand, Oppennoorth and Asperen¹⁰ proposed that the detoxication in some organophosphate-resistant strains of house flies was due to "mutant ali-esterases". These enzymes are inhibited by selective ali-esterase inhibitors such as *n*-propyl paraoxon^{8,10} and the oxo analog of EPN⁹, which show synergism with malathion against resistant strains. Menzel *et al.*¹¹ tried to separate housefly esterases by starch gel electrophoresis. Their studies indicated marked differences among various strains including susceptible and malathion resistant strains but no characteristic pattern of resistance was observed.

In the present work, the esterases of malathion resistant houseflies and green rice leafhoppers were separated by agar gel electrophoresis and the correlation between malathion degrading enzymes and specific esterases inhibited by phosphoric esters having synergistic action to malathion was studied.

† Present address: Faculty of Agriculture, Meiji University, Kawasaki

Materials and Methods

Insects: The following houseflies were used;

(1), Takatsuki (*Musca domestica vicina* Macquart), a susceptible strain; (2), Hokota (*Musca domestica vicina* Macquart), a diazinon-resistant strain showing cross-resistance to malathion, given by Dr. Z. Ogita, Osaka University. (3), G (*Musca domestica* L.), a malathion resistant strain, given by Dr. F. Matsumura, University of Wisconsin. The strains of green rice leafhoppers (*Nephotettix cincticeps* Uhler) were: (1) Odawara, a susceptible strain and (2) Koti, a malathion resistant strain. This was used after further selection with malathion for several generations.

Chemicals: *O, O*-dimethyl *S*-(1, 2-bis-carbophenoxy) ethyl phosphorodithioate (carbophenoxy malathion) was synthesized from *O, O*-dimethyl phosphorodithioate and phenyl fumarate in the presence of a little amount of hydroquinone. P^{32} -labeled malathion was prepared from P^{32} -labeled diphosphorus pentasulfide according to Krueger and O'Brien¹²⁾ and purified by silica gel column chromatography. Paraoxon and *n*-propyl paraoxon were prepared from *p*-nitrophenyl phosphorodichloridate and corresponding alcohol by the action of pyridine. Oxo analog of EPN (EPNO) was obtained from EPN by oxidation with bromine water and purified by silica gel column chromatography. The saligenin cyclic phosphorus esters, 2-methoxy-4H-1, 3, 2-benzodioxaphosphorin-2-oxide (K-7) and 2-phenoxy-4H-1, 3, 2-benzodioxaphosphorin-2-oxide (K-2), were synthesized by the reported method.^{1, 2)}

Pesticidal tests: The test methods were previously reported⁹⁾. For green rice leafhopper, a microtechnique reported by Kojima *et al.*¹³⁾ was used. Joint toxicity was tested by applying the mixture of malathion and synergist at 1:1 ratio and evaluated by the cototoxicity coefficient of the mixture.

cototoxicity coefficient =

$$\frac{LD_{50} \text{ of malathion alone}}{LD_{50} \text{ of malathion in a mixture}}$$

Agar gel electrophoresis: Agar gel electrophoresis was performed by the method of Ogita^{14, 15)} as described in the previous paper⁶⁾. β -Naph-

thyl acetate was used as a substrate of esterases. Whole houseflies were homogenized in deionized water at the concentration of 3 g flies per ml. The homogenate was centrifuged at 10,000 r.p.m. for 1 hr. and the supernatant was used. Six females of green rice leafhoppers were crushed in 0.1 ml of deionized water on a glass plate and the resulted brei was placed on an agar gel layer using a strip of filter paper, 2 mm $\times$ 10 mm. For the preparation of enzyme solution, agar gel plate with 4 mm thick was used. A part of the plate was stained to detect esterases and agar gel blocks of corresponding esterases were cut out, frozen, thawed and extracted with 1% saline. Protein was stained with bromothymol blue (BTB) and extracted with weak alkaline solution. The absorption of the resulted solution was measured at 595 $m\mu$.

Assay of malathion-carboxyester degrading enzymes: Carbophenoxy malathion was introduced as a substrate for the determination of enzyme activity degrading malathion-carboxyester. The enzyme activity could easily be estimated by the photometric determination of produced phenol. Whole insects were homogenized in 1% saline at one insect per ml. The homogenate was centrifuged and the resulted supernatant was used for the assay. The enzyme solution extracted from agar plate was also used. The standard enzyme assay method was as follows: One ml of the enzyme solution was incubated with 1 mg of carbophenoxy malathion in 0.1 ml acetone for 30 min. at 21°C. After incubation, 4 ml of M/15 phosphate buffer of pH 7.4, 3 ml of 0.05% 4-aminoantipyrine and 2 ml of 0.4% potassium fericyanide was added in order. The absorption at 510 $m\mu$ was measured with a Hitachi spectrophotometer.

Assay of malathion degrading enzyme: Matsumura's technique using P^{32} -malathion was also applied for the assay of malathion degrading enzymes. The enzyme solution extracted from agar gel plate was incubated with 200 μ g of P^{32} -malathion in 0.1 ml of ethanol for 1 hr. at 23°C. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid and pH was adjusted to 2 with 1 N sodium hydroxide. The carboxy-esterase products and unreacted malathion were

extracted with two portions of 5 ml chloroform and malathion-products having free carboxylic acid group were re-extracted from the chloroform layer with 10 ml of M/15 phosphate buffer of pH 7.0. The aqueous extracts of P^{32} were assayed by means of GM-tube, after drying 1 ml of aliquots in metal planchets.

Results

Synergism of K-2 with malathion

Susceptibilities to malathion were assessed by means of topical application. When a susceptible strain, Takatuki, was cultured under the pressure of malathion for 12 generations, the resistance to malathion of this strain was slightly enhanced. The levels of resistance to malathion of two strains, Hokota and G, as compared with the susceptible strain Takatuki, were found to be 4 and 20-fold respectively. A phenyl derivative of saligenin cyclic phosphorus ester, K-2, increased the toxicity of malathion 9.2 to 10.8 times against both resistant strains (Table 1). K-2 is, therefore, a quite effective synergist of malathion against resistant strains of houseflies.

Table 1. Toxicity of malathion against some strains of house flies with or without synergist saligenin cyclic phenyl phosphate (K-2).

strain	LD ₅₀ malathion (μ g/♀)		cotoxicity
	malathion alone	mixed with K-2 (1:1)	
Takatuki	0.6		
*Selected Takatuki	0.8		
Hokota	2.5	0.27	9.2
G	12.5	1.16	10.8

* selected with malathion for 12 generations

Inhibition of housefly esterases by synergists of malathion

Housefly esterases were separated by agar gel electrophoresis. The marked differences were found between esterase zymograms of three strains of houseflies. The susceptible strain, Takatuki, showed six esterase bands hydrolyzing β -naphthyl acetate. The esterases of Hokota were separated into four bands, and 1st and 4th bands from anode were inhibited by K-2 (Fig. 1). The resistant strain G contained seven esterase bands (Fig. 2). Among of them, 1st,

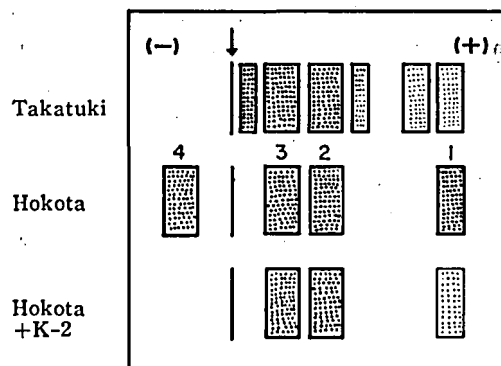


Fig. 1. Esterase zymograms of houseflies. K-2 (10^{-5} M) was incubated with enzyme solution for 30 min. at 0°C before electrophoresis.

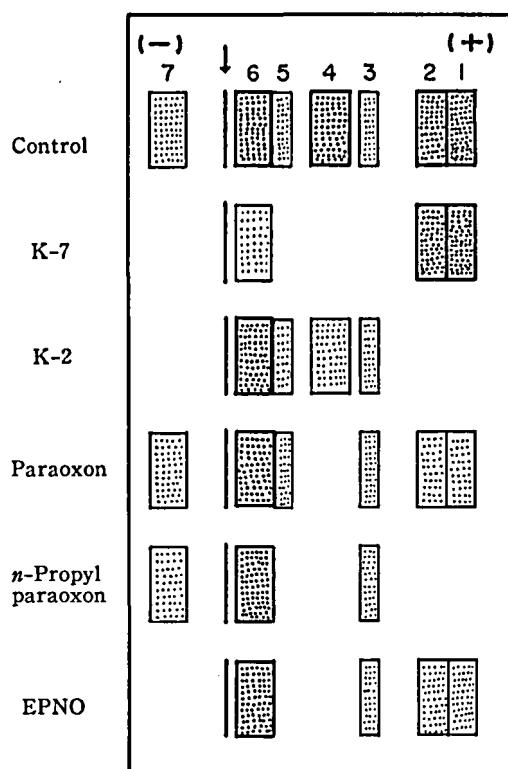


Fig. 2. Effect of organophosphorus esters on esterase zymograms for G strain. Inhibitors were incubated with enzyme solution for 30 min. at 0°C before electrophoresis. K-7: 10^{-5} M. K-2, paraoxon, *n*-propyl paraoxon and EPNO: 10^{-5} M.

2nd and 7th bands disappeared specifically by the treatment with K-2, while 3rd to 7th bands

vanished with K-7. Furthermore, the specificity in G strain esterase inhibition of some phosphoric esters which have been reported as synergists of malathion was examined in comparison with K-2. EPNO inhibited completely 4th, 5th and 7th bands and inhibited slightly 1st and 2nd bands. *n*-Propyl paraoxon inhibited 1st, 2nd, 4th and 5th bands but did not inhibit 7th band. While paraoxon inhibited 1st, 2nd and 4th bands (Fig. 2). The esterases which disappeared by the treatment with K-2 were found to be inhibited by the action of either EPNO or *n*-propyl paraoxon. It seems that these esterases may concern with the malathion degrading enzymes.

Malathion-carboxyester degrading activity of housefly esterases

The enzyme activity to degrade malathion-carboxyester was assessed by means of carbophenoxy malathion as a substrate. The results are shown in Table 2. The enzyme activity of Takatuki strain increased slightly by culture

Table 2. Enzyme activity degrading carbophenoxy malathion of housefly strains.

strain	degradation ($\mu\text{g}/\text{fly}$)
Takatuki	2.4
Selected Takatuki	2.9
Hokota	4.4
G	3.0

Degradations are expressed as $\mu\text{g}/\text{fly}$ of produced phenol for 30 min.

under the pressure of malathion for 12 generations. The enzyme activities of two resistant strains, Hokota and G, were higher than that of the susceptible strain. However, the enzyme activities hydrolyzing malathion-carboxyester of these fly strains are not parallel with the levels of resistance to malathion. The level of resistance to malathion of G strain was about 20-fold as compared with Takakuki, but carboxyester degrading activity of the former was only 1.25 times higher than that of the latter (Table 2).

The esterases of Hokota were separated into four fractions by agar gel electrophoresis and the activity to degrade carbophenoxy malathion of each fraction was estimated. As shown in

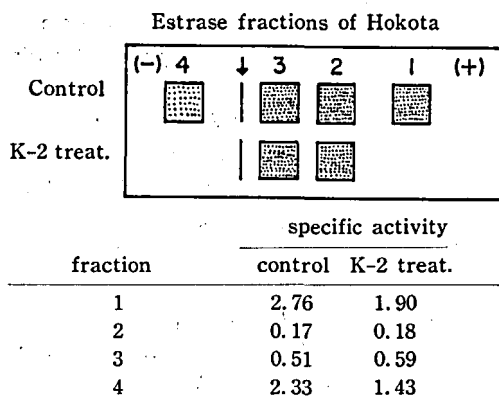


Fig. 3. Degradation of carbophenoxy malathion by esterase fractions from Hokota strain.

Specific activity was expressed in E_{510}/E_{595} .

E_{510} : absorption of phenol reacted with 4-aminoantipyrine at 510 $m\mu$.

E_{595} : absorption of protein stained with B.T.B. at 595 $m\mu$.

Fig. 3, the high activity was observed in the fractions 1 and 4. The activities in both fractions were lowered to near one-half of their original activities by the action of 10^{-6}M of K-2. This agrees well with the fact that K-2 inhibits the 1st and 4th bands of esterases to hydrolyze naphthyl acetate as shown in Fig. 1. It seems that synergistic effect of K-2 on Hokota may be due to the inhibition of 1st and 4th esterase fractions to hydrolyze malathion-carboxyester.

Carboxyesterases degrading malathion in G strain

The esterases of G strain were separated three fractions, A, B and C, by electrophoresis with agar gel of 4 mm thick. Fraction A corresponded to 1st and 2nd bands, fraction B to 3rd, 4th, 5th and 6th bands, and fraction C to 7th band in Fig. 2. This means that the esterase activities of fraction A and C are inhibited by the preincubation with 10^{-6}M of K-2 (Fig. 2). Each esterase fractions extracted was applied for the assay of the enzyme activity to hydrolyze P^{32} -malathion. Malathion degrading activity was found in both fractions A and C, but not in B. Fraction C was more active than A. Thus, carboxyesterases degrading malathion distribute in band 7 and 1 or/and 2. The malathion degrading activity of both fractions was completely inhibited with K-2

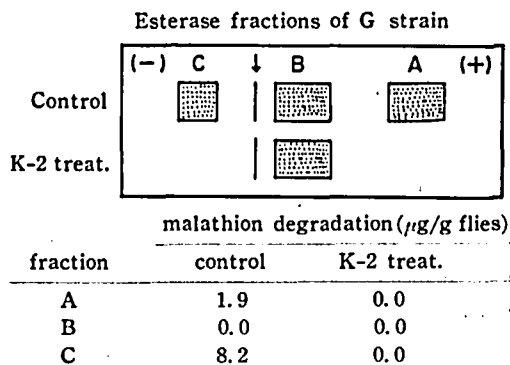


Fig. 4. Degradation of P^{32} -malathion by esterase fractions from G strain.

The activity expressed as $\mu\text{g/g}$ flies conversion of 200 μg of P^{32} -malathion incubated with each fraction for 60 min.

at 10^{-6}M (Fig. 4). The inhibitory activity of K-2 for these enzymes would be responsible for its synergism with malathion. As shown in Fig. 2, esterase bands 1, 2 and 7 were also inhibited by known organophosphorus synergists such as EPNO or *n*-propyl paraoxon.

Relation between malathion-carboxyester degrading enzymes and esterases in resistant strain of rice leafhopper

Susceptibilities of green rice leafhoppers to malathion were assessed by means of a micro technique. The level of resistance to malathion of Koti, as compared with a susceptible strain Odawara, was about 65-fold. The synergistic effect of K-2 on two strains was tested. The synergism of K-2 against Odawara and Koti was 2 and 4.6-fold respectively (Table 3). As in the case of houseflies, K-2 is an effective synergist of

Table 3. Synergism of saligenin cyclic phosphorus esters with malathion on green rice leafhoppers.

strain		LD ₅₀ ($\mu\text{g}/\text{♀}$)	cotoxicity
Odawara	malathion alone	0.003	2.0
	malathion+K-2 (1:1)	0.003	
Koti	malathion alone	0.196	4.6
	K-7 alone	0.015	
	K-2 alone	0.147	
	malathion+K-7 (1:1)	0.030	
	malathion+K-2 (1:1)	0.087	

malathion against resistant strain.

The esterases of two strains were compared by means of agar gel electrophoresis. The susceptible strain Odawara showed two feeble esterase bands, while the resistant strain Koti exhibited two active bands which disappeared completely by the treatment with either K-2 or K-7 at 10^{-6}M (Fig. 5).

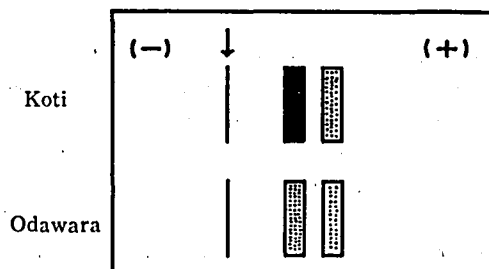


Fig. 5. Esterase zymograms of green rice leafhoppers.

Odawara: susceptible strain
Koti: malathion-resistant strain

The enzyme activity degrading malathion-carboxyester was estimated by the use of carbo-phenoxy malathion as a substrate. The activity of Koti was 1.8 times higher than that of Odawara (Table 4). The esterase fraction ascertained with electrophoresis was applied for the assay of the enzyme activity degrading malathion-carboxyester. The results presented in Table 5 show that both K-2 and K-7 inhibited the enzyme

Table 4. Enzyme activity degrading carbo-phenoxy malathion of green rice leafhoppers.

strain	degradation ($\mu\text{g}/\text{♀}$)
Odawara	4.0
Koti	7.3

Degradations are expressed as $\mu\text{g}/\text{♀}$ of produced phenol for 30 min.

Table 5. Inhibition of carbophenoxy malathion degrading enzyme from Koti strain.

	inhibition (%)
K-2 $2 \times 10^{-6}\text{M}$	33
K-7 $2 \times 10^{-6}\text{M}$	45

After electrophoresis, esterases extracted with 1% saline were incubated with inhibitors at 21°C for 30 min.

activity to hydrolyze malathion-carboxyester. It seems that these esterases concern with malathion degrading enzymes and synergism of K-2 with malathion may be at least partially due to the inhibition of these enzymes. These enzymes were also inhibited with K-7. Ineffectiveness of K-7 in synergism should be owing its high toxicity (Table.3).

Discussion

Malathion may be detoxified by either phosphatase action or esterase action. The low toxicity of malathion to mammals is explained by the latter mechanism.¹²⁾ The resistance to malathion is also caused by the carboxyesterase in some species of insects.^{7,8)} The inhibition of detoxication of malathion may result in synergism. It has been found that carboxyesterase is highly sensitive to inhibition by EPNO and *n*-propyl paraoxon⁹⁾, which are known as synergists of malathion.

The phenyl derivative of saligenin cyclic phosphorus ester (K-2) synergized the insecticidal action of malathion against houseflies and green rice leafhoppers, especially malathion resistant strains of both insects. It is reasonable to think that synergistic action of K-2 is caused by the inhibition of carboxyesterase degrading malathion.

Menzer *et al.*¹¹⁾ tried to separate esterases from six strains of houseflies by means of starch gel electrophoresis. Ogita and Kasai¹⁵⁾ reported that esterases of houseflies were separated into many bands by agar gel electrophoresis. However, the neither experiments using β -naphthyl acetate as a substrate found any characteristic esterases with respect to resistance. When P³²-malathion and carbophenoxy malathion were used, malathion degrading enzymes were found to concern with esterases hydrolysing β -naphthyl acetate. Nevertheless, the intensity of former enzyme activity was not paralleled to that of latter enzymes. In the case of G strain, the esterase activities of fraction A and B were higher than that of fraction C, while the malathion degrading activity of fraction C was highest among three fractions. This is, of course, due to the difference in the substrate specificity of these enzymes.

Dauterman and Main¹⁶⁾ reported the relative

enzymatic activity of rat liver carboxyesterase toward malathion homologs. In vitro studies showed relative enzymatic activity to be highest for the carbomethoxy malathion and to decrease with an increase in chain length of the carboalkoxy group. Carbophenoxy malathion used in the present experiment have neither insecticidal activity nor synergism with malathion against houseflies and green rice leafhoppers. It is thought to be readily degraded with various esterases. Although the degradation of this substrate with esterases from two insects was high enough to detect the enzyme activities, correlation between carbophenoxy malathion degrading activity and resistance to malathion was clear in neither insects. This appears to be partly due to the high substrate specificity of carboxyesterases degrading malathion in resistant strains.

There are at least two enzymes to degrade malathion. One has high electrophoretic mobility toward anode and another one moves toward cathode. The former is relatively non-specific to substrate; both naphthyl acetate and carbophenoxy malathion are good substrates but malathion is rather poor substrate. The latter enzyme is rather specific to malathion; naphthyl acetate and carbophenoxy malathion are poor substrates. The non-specific enzyme exist in susceptible strain too and increases in resistant strains. However, the specific one appears only in resistant strains. The contribution of the latter to resistance is much more than that of the former.

Ozaki and Koike¹⁷⁾ reported that malathion resistant green rice leafhoppers had significantly higher esterase activity to hydrolyze β -naphthyl acetate than susceptible ones had. Kasai and Ogita¹⁸⁾ suggested that there was well correlation between malathion resistance and esterase activity of a certain band. It is thought that malathion resistance of green rice leafhopper is probably owing to the action of esterases which have an ability to hydrolyze both β -naphthyl acetate and carbophenoxy malathion. Synergism of K-2 with malathion may be at least partly due to the inhibition of these esterases as in the case of houseflies.

Some aryl derivatives of saligenin cyclic phosphate are synergistic with malathion against susceptible mites too. However, the synergistic effect is much low in resistant strains in contrast to the above mentioned insects.¹⁹⁾ Smissaert²⁰⁾ suggested that the mechanism of resistance to phosphorus esters in the resistant strain of mites is not the increased detoxication of the phosphorus esters, but the decreased inhibition rate of its cholinesterase.

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Summary

The saligenin cyclic phenyl phosphate (K-2) showed high synergistic activity to malathion, particularly against resistant strains of house flies and green rice leafhopper. The esterases from malathion-resistant houseflies and green rice leafhopper were separated by agar gel electrophoresis. These esterases were characterized by inhibition of organophosphorus esters having synergism with malathion such as EPNO, *n*-propyl paraoxon and K-2. At least two esterases inhibited by these phosphate esters showed to have an ability to hydrolyze carbophenoxy malathion or malathion. One is more specific to malathion and another is relatively non-specific. These esterase activities are much higher in resistant strains than in susceptible strains. The synergistic action of K-2 with malathion is thought to be mainly due to the inhibition of carboxyesterase degrading malathion.

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