Naphthyl acetate esterase in the green rice leafhopper, *Nephotettix cincticeps* UHLER, with special reference to the resistant colony of the organophosphorus insecticide.

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INTRODUCTION

The green rice leafhopper, *Nephotettix cincticeps* UHLER, had been well controlled by malathion in Kochi Pref. since 1953, but in 1961, it was reported that malathion failed to control in some parts of Kochi Pref. OZAKI found that the leafhopper had developed a malathion resistance in Ōsone area. By later tests on the susceptibility to malathion and methyl parathion, it was authenticated that the malathion or methyl parathion resistance colony inhabited various areas of Japan (OZAKI, in press). OZAKI and KUROSU (in press) demonstrated also that the malathion and methyl parathion resistant colonies crossed to some other organophosphorus insecticides. In consideration of the above results, the attention is concentrated on the biochemical mechanisms in the development and genetics of insecticide resistance in the leafhopper.

KOJIMA *et al* (1963) investigated the biochemical mechanisms of development of the resistance to malathion and found that the increase in the activity of carboxy-esterase was a cause of the development of resistance, but the cholinesterase activity and susceptibility of the esterase to malaoxon were not connected with the development of malathion resistance.

The primary objective of this study is to assay of the esterase in the leafhopper with an aid of β -naphthyl acetate as substrate, and to examine the difference between the esterase activity in susceptible and organophosphorus insecticide resistant leafhopper.

MATERIALS AND METHODS

Insects The eggs or larvae of the leafhopper were collected from Konosu, Soka and Kasukabe in Saitama Pref. and Koga in Ibaraki Pref. where the total amounts of insecticides which had been used to control the rice pests were very little, and from Ōsone in Kochi Pref., Nakagawahara in Ehime Pref., Okayama in Okayama Pref. and Oyama in Tochigi Pref., where the leafhopper had been known develop resistance to organophosphorus insecticide. The eggs or larvae collected were reared under sixteen hours illumination at 25°C. The larvae and adults were used throughout the experiments.

Enzyme solution Whole homogenates were prepared by homogenation of the insects in definite volumes of phosphate buffer (M/15, pH 7.0) using glass homogenizer of Potter-Elvehjem type. The resultant homogenate was centrifuged for 5 min at 3500 r. p. m. at 4°C, and the supernatant was used as enzyme solution.

Substrate solution β -naphthyl acetate (Tokyo Kasei Co.) was used as substrate. The substrate solution was prepared as follows. Ten mg of the β -naphthyl acetate was dissolved in 2 ml of acetone and added to 50 ml of the phosphate buffer of pH 7.0, then filled to 100 ml with distilled water.

The experimental procedures followed the method of NACHLAS and SELIGMAN (1949) with slight modification. After preincubation of substrate solution for 5 min., 5 ml of this solution and 1 ml of the enzyme solution were incubated for 5~20 min at

37°C. At the end of the incubation period, 1 ml of the solution containing 4 mg of naphthanil diazo blue B (Tokyo Kasei Co.) was added and well shaken. After a few min., 1 ml of 40 per cent trichloroacetic acid was added. The coupled diazotized red color was extracted with 10 ml of ethyl acetate. After centrifugation at 3500 r.p.m. for 5 min., absorbance of ethyl acetate extract was measured by colorimetric method with a 540 m μ filter.

RESULTS

Standard curve for the determination of β -naphthol The relation between the amount of β -naphthol liberated by the esterase action and the absorbance at 540 m μ are shown in Fig. 1.

Fig. 1. Standard curve for the determination of β -naphthol.

As shown in Fig. 1, a straight line was obtained between 0 and 50 μ g of β -naphthol.

Optimum pH for the esterase activity The influence of pH on the esterase activity of homogenate to β -naphthyl acetate were studied by changing pH of the phosphate buffer.

It is shown in Fig. 2, that the optimum pH range is from 6.8 to 7.0. Non-enzymatic decomposition of substrate was remarkable at alkaline pH. This is mainly

Fig. 2. Influence of pH on the esterase activity. Insect; Oyama colony. Homogenate concentration; adult/5 ml buffer. Reaction time; 5 min.

Fig. 3. Influence of reaction time on the esterase activity. Insect; R and S indicates Oyama colony and Konosu colony respectively. Homogenate concentration; adult/5 ml buffer of pH 7.6.

influenced by remarkable coupling of diazotized red color product.

Reaction time The enzyme solution was incubated with substrate-buffer solution for various period, and the relation between reaction time and rate of hydrolysis were studied. The results obtained are given in Fig. 3.

The esterase activity increases linearly with a period of incubation until 20 min. both in the malathion resistant and susceptible leafhopper. But in the malathion resistant Oyama colony increase in the activity becomes slower after 20 min. The malathion resistant leafhopper is more active than the susceptible one and the female is more active than the male.

Effect of enzyme concentration on the esterase activity The relationship between the esterase activity and the concentration of the enzyme were investigated. The results obtained are shown in Fig. 4.

As shown in Fig. 4, the esterase activity increases linearly with increase of the

Fig. 4. Influence of enzyme concentration on the esterase activity. Insect; R and S indicate Oyama and Konosu colony respectively. Reaction time; 5 min. pH; 7.0.

enzyme concentration. Degree of activity in the malathion resistant leafhopper is higher than that in the susceptible one.

Changes of the esterase activity during larval growth and difference in sexes The esterase activity was assayed in larvae and adults of malathion resistant Ōsone colony and susceptible Konosu colony. The results obtained are shown in Fig. 5.

Fig. 5. The esterase activity in larvae and adults of the leafhopper. Solid bars are malathion resistant Ōsone colony, hollow bars susceptible Konosu colony. Reaction time; 20 min. pH; 7.0.

In larvae, the esterase activity is increased according to the progress of instars. Adults have higher activity of esterase than larvae. In 5th instar larvae and adults, the esterase activity in female is more than that in male. These differences among instars, stages or sexes in esterase activity are caused not only by the increased body weight but by other factors such as the nature of enzyme proteins. The increased rate of activity with changes in the instar and the stage is higher in the malathion resistant leafhopper than in the susceptible one. In malathion resistant leafhopper, the esterase activity shows remarkable differences between 5th instar larvae and adults.

No such a wide difference is found in the susceptible leafhopper.

Relation between the esterase activity and resistance to organophosphorus insecticide As mentioned above, the esterase activity showed the significant difference between the malathion resistant leafhopper and the susceptible one. Still, it was not clarified whether the variation in its activity was connected with the development of malathion resistance of the leafhopper or other insecticide resistance. Then further investigation was made to ascertain the esterase activity and susceptibility to malathion and methyl parathion with the colonies collected from various localities.

Table 1 shows the esterase activity of various colonies. The highest activity was observed in Oyama colony, next was Nakagawahara, these were followed by Kuroda and Okayama colony.

Table 1. Difference of the esterase activity among colonies.

Colony	Homogenate concentration (adult/buffer)	Optical density
Oyama	1/5	2.000
Kuroda		0.775
Koga		0.137
Soka		0.182
Kasukabe		0.103
Konosu		0.166
Okayama		0.673
Oyama	1/10	1.229
Nakagawahara		1.018

Reaction time; 20 min. pH; 7.0.

The LD-50 of individual colonies to malathion and methyl parathion is shown in Table 2. Tables 1 and 2 show that there is a high correlation between the esterase activity and LD-50 to malathion.

In the Oyama colony, which gave the highest activity of the esterase among all the test colonies, LD-50 to methyl parathion was lower than in Nakagawahara or in the Okayama colony. No relationship between the esterase activity and the LD-50 to methyl parathion was found. Consequently, it is considered that the

Table 2. LD-50 of malathion and methyl parathion applied topically on the leafhopper female adults of various colonies. (OZAKI and KUROSU, in press)

Colony	LD-50 in μg per gram of body weight	
	Marathion	Methyl parathion
Oyama	10.13	48.37
Konosu	0.65	6.84
Okayama	3.51	141.98
Nakagawahara	12.41	319.76

striking increase on the esterase activity takes part in malathion resistance of the leafhopper.

The *in vitro* esterase inhibition with malaoxon and paraoxon The *in vitro* inhibition experiments to the esterase were carried out with various doses of malaoxon and paraoxon. The relation between the inhibition rate and the amount of inhibitor gave sigmoid curves. From these curves, ID-50 values of malaoxon and paraoxon for esterase were obtained as Table 3.

The ID-50 of malaoxon ranged from 3.57×10^{-7} M to 5.01×10^{-7} M and that of paraoxon ranged from 7.87×10^{-10} M to 8.63×10^{-10} M. The ID-50 values of malaoxon and paraoxon for the esterase of malathion resistant colony were approximately the same as those of susceptible colony.

The *in vitro* esterase inhibition with eserine and DFP In the susceptible Konosu colony and the malathion resistant Ōsone

Table 3. ID-50 of malaoxon and paraoxon for the esterase.

Colony	Sex	ID-50 (M)	
		Malaoxon	Paraoxon
R	♂	3.57×10^{-7}	7.87×10^{-10}
R	♀	4.16×10^{-7}	8.63×10^{-10}
S	♀	5.01×10^{-7}	8.24×10^{-10}

Insect; R and S are Oyama and Konosu colony respectively. Homogenate concentration; female of R, 1/20, male of R and female of S, 1/10. Reaction time; 20 min. Incubation with inhibitor; the homogenate was incubated with each final concentration of inhibitor for 30 min at 37°C.

Fig. 6. Inhibition rate of the esterase activity with eserine and DFP. Homogenate concentration; female of Oyama colony, 1/20, male of Oyama and Ōsone colony, 1/10, female of Konosu colony, 1/5, male, 1/3. Reaction time; 20 min. pH; 7.0. Incubation with inhibitor; as in explanation of Table 3.

and Oyama colony, *in vitro* inhibition experiments for the esterase were conducted with eserine and DFP. The experimental results are given in Fig. 6.

The inhibition rate by 10^{-5} M eserine was ranged from 66 per cent to 78 per cent. This value of inhibition rate is comparable to that of kidney, pancreas and liver of dog and human (NACHLAS and SELIGMAN, 1949). The esterase was inhibited more than 97 per cent by DFP at 10^{-5} M. Significant differences in inhibition by DFP or eserine among the colonies were not obtained.

DISCUSSION

The method of naphthyl acetate esterase determination and the difference between

susceptible and organophosphorus insecticide resistant leafhopper were studied in this report. The method of determining esterase activity by using naphthyl esters as substrate was established by NACHLAS and SELIGMAN in the mammalian tissue homogenates. KOIKE (in press) found that this method is so sensitive and specific for insects, and applicable to small insects such as aphid. VAN ASPEREN (1963) reported a method that esterase activities can be measured in the range of 10 μ g and 100 μ g of the housefly, and he applied it to the determination of the esterase activity in individual adults of spider mite (*Tetranychus urticae*).

From the results reported in this paper it was found optimum that the enzyme concentration was from 5 to 20 ml of buffer per adult and the reaction time was from 5 to 20 min. The increase of esterase activity was connected closely with the development of resistance to malathion in the leafhopper, but not with that to methyl parathion.

As above, the esterase activity in the leafhopper is assayed speedily and individually, and increase remarkably in the malathion resistant colony. By determining the esterase activity it may be able to examine whether or not a leafhopper develops malathion resistance, and this method has the biological significances that the resistance mechanisms or the heredity of the resistance to malathion may be analyzed enzymologically.

According to the results obtained by VAN ASPEREN, no significant differences were found between the esterase activities of susceptible and *o-p* resistant houseflies when α - and β -naphthyl acetate used as substrate. But using α -naphthyl acetate, the non-eserine inhibited esterase was more active in *o-p* resistant strain than that in susceptible strain. No such difference was found when β -naphthyl acetate was used. While the esterase activity in the leafhopper was much higher for a malathion resistant colony than for a susceptible one, the ratio of the non-eserine inhibited

esterase activity to total esterase activity in both colonies of susceptible and malathion-resistance gave a somewhat similar activity when β -naphthyl acetate was used as substrate.

KOJIMA *et al* (1963) suggests that the resistance mechanisms of insects to malathion are divided into following two types, that is, one caused with the increase of phosphatase activity and the decrease of B-esterase activity, as in the case of housefly; the other caused with the increase of carboxy esterase activity as in the case of mosquitoes. They suggest that the resistance mechanisms to malathion in the leafhopper belong to the latter case.

By using a selective inhibitor, esterases were separated into cholinesterase, B-esterase and A-esterase. The ratio of B-esterase activity in malathion resistant leafhopper was not seem to be different from that of susceptible one (see Fig. 6). As pointed by KOJIMA *et al*, the resistance mechanisms to malathion in the leafhopper differ markedly from that of the *o-p* resistant housefly, in which B-esterase activity becomes lower than in the susceptible one (VAN ASPEREN, 1962). This dissimilation on the resistance mechanisms is due to the differences in the species of insects.

In this insect, activity of cholinesterase is not altered by the development of resistance to malathion (KOJIMA *et al*, 1963). But as shown in Figs. 5 and 6, the cholinesterase for the malathion resistant leafhopper was more active than that for susceptible leafhopper. Conclusively, in the malathion resistant leafhopper, the esterases hydrolysing malaoxon or malathion are higher activity; on the other hand the activities of these esterase are attended by increases of cholinesterase and other esterases activities.

The results reported here show that the increase of the naphthyl acetate esterase activity affects the development of malathion resistance in the leafhopper; therefore it is suggested that the increased

parts of the esterase activity in malathion resistant leafhopper may include the esterase hydrolyzing malaoxon or malathion.

SUMMARY

Optimum conditions for determination of esterase in the green rice leafhopper was determined by using β -naphthyl acetate as substrate, and the difference between esterase activity of susceptible and organophosphorus resistant leafhopper was examined.

This method was very sensitive, and it was found that the optimum concentration of homogenate was from 5 ml to 20 ml of buffer per adult and the reaction time was from 5 min. to 20 min. In larva, the esterase activity increased with age, the adult generally showed higher activity than larva and female was higher than male.

The malathion resistant leafhopper had significantly higher activity of esterase than the susceptible one. Increase in esterase activity was closely correlated with malathion resistance. The ID-50 values of malaoxon and paraoxon for the esterase of malathion resistant colony were approximately the same as those of the susceptible colony. In both the resistant and susceptible leafhopper, the esterase activity consisted of cholinesterase and B-esterase. The ratio of each esterase activity to total esterase activity was not differed between the resistant and the susceptible leafhopper.

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摘 要

ツマグロヨコバイにおけるナフチルアセテートエステラーゼ，とくに有機りん剤に抵抗性の個体群における異常な高活性について

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基質に β -naphthyl acetate を用いてツマグロヨコバイのエステラーゼ活性を測定する方法を検討し，このエステラーゼ活性が有機りん剤に抵抗性の個体群と感受性の個体群とで異なるかどうかを調べた。

基質に β -naphthyl acetate を用いた場合，エステラーゼ活性の測定感度は極めて高く，成虫の 1 個体を 5 ml から 20 ml のりん酸緩衝液で磨砕した酵素液で測定することができ，また反応時間は 5 分から 20 分の間で十分であった。なお最適 pH は 6.8 から 7.0 の範囲であった。

幼虫のエステラーゼ活性は生育するにつれて増大した。成虫のエステラーゼ活性は幼虫より高く，雌は雄より高かった。

馬拉ソンに抵抗性の個体群では，感受性の個体群に比べてエステラーゼ活性は有意に高かった。各地から採集

した個体群の間にはエステラーゼ活性に顕著な違いがみられたが，エステラーゼ活性と馬拉ソンに対する LD-50 との間には高い相関関係があった。しかし，メチルパラチオンに対する LD-50 との間には同様な関係はみられなかった。ツマグロヨコバイにおけるナフチルアセテートエステラーゼ活性の増大は馬拉ソンに対する抵抗性の発達に関係するものと考えられる。

ナフチルアセテートエステラーゼに対する マラオクソンとパラオクソンの ID-50 は馬拉ソンに抵抗性の個体群と感受性の個体群との間で差がなかった。ナフチルアセテートエステラーゼ活性は コリンエステラーゼと B-エステラーゼからなっていた。合計エステラーゼ活性に対する各エステラーゼ活性の割合は抵抗性個体群と感受性個体群の間で差がなかった。