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ESTERASE OF DIAMOND-BACK MOTH (*PLUTELLA XYLOSTELLA* L.). I. ENZYMATIC PROPERTIES OF LARVAL ESTERASES[1]

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William Can-Jen Maa and Mei-Ling Chuang (1983) Esterase of diamond-back moth (*Plutella xylostella* L.). I. Enzymatic properties of the larval esterases. *Bull. Inst. Zool., Academia Sinica* **22**(2): 123-131. Hydrolytic activity of 1-naphthylacetate catabolized by larval esterases of diamond-back moth (*Plutella xylostella* L.) was investigated. The optimal conditions for esterases were determined. Reaction of the esterase with the substrate in 0.1 M potassium phosphate buffer of pH 7.5 under 27°C for 15 minutes, showed the greatest activity.

The non-specific esterases characterized by inhibitors, was found to be mainly the aliesterases. Increasing protein content to the enzyme mixture showed no effect to the esterase activity. Alcohol and acetone solution either enhanced or inactivated the esterase activity. Storage of the enzyme under −80°C for several months showed no effect to the esterases. The activity decreased rapidly when the enzyme was stored under 0° or −20°C.

The apparent K_m for the substrate was $(3.5\pm0.4)\times10^{-5}$ M and V_{max} was $(6.1\pm0.2)\times10^{-6}$ M. Study on subcellular distribution of the esterase activity revealed that approximately 68% of the activity was in the soluble fraction and 26%, in all the membraneous fractions. However, when enzyme specific activity was accounted for the activity of these fractions which ranked in an order of microsome, mitochondria, soluble fraction, and cell debris. The possible role of the larval esterases that played on pesticide degradation was discussed.

Insect esterases are a poorly defined group of enzyme with both protective and physiological roles. They catalyze the hydrolysis of various groups of insecticides, insect growth regulators, juvenile hormones, and insect pheromones and are thus of considerable important in insecticide action as well as the growth, development, and behavior of insects. The mechanism of the degradation of the synthetic esters, like malathion, carbamates, pyrethoids, juvenile hormone analogs, and pheromones, the resistance to these compounds by insect species, of ecomonic importance, has been intensively investigated recently years (Ahmad, 1976; Mayer *et al.*, 1982).

In vivo study on pesticide resistance of the diamond-back moth, the most notorious vegetable pest in Taiwan, has already been studied by Liu *et al.* (1982). They suggested that the degradative activity of the larval esterase (*s*) was associated with the resistance mechanism of the insect (Sun *et al.*, 1978), although the *in vitro* information about the esterase (*s*) was not clear.

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It is necessary that a serious effort be made to gain a better understanding of the properties of the esterase system of this insect, otherwise further advances in the study of the moth esterases will be hampered. This study is to investigate the quantitative *in vitro* information of the larval esterase with following aspects: (1) What catagories of the diamond-back moth larval esterase was possibly involved in the pesticide-resistance? (2) What is the optimal conditions for the esterase reaction? (3) How is the stability of the esterases under various physical and chemical imposes? (4) In an aspect of pesticide detoxication of the diamond-back moth, what is the significance of the subcellular distribution of the larval esterases?

MATERIALS AND METHODS

Insects

Diamond-back moth, *Plutella xylostella* (L.), collected from vegetable farms at Bamboo Lake (BL), Taipei, were reared in insectarium according to Koshihara and Yamada (1976), and Maa *et al.* (1983). The fifth instar larvae of the moth were used as test insect. Insect populations from Geou-Fang (GF), I-Lan (IL), and C-Hou (CH) were also used.

Chemicals

All chemicals and reagents were of analytical grade or the best grade available. Paraoxon (Diethyl-*p*-nitrophenyl phosphate), 96% purity, was purchased from Chem Service, Inc. Eserine (1, 2, 3, 3a, 8, 8a-hexahydro-1, 3a, 8-trimethylpyrrolo-indol-5-ol methylcarbamate), para-hydroxy mercuribenzoate (PHMB), lauryl sulfate, diazoblue, Brilliant Blue G, 1-naphythylacetate (NA), Bovine Serum Albumin (BSA), and all the other chemicals were purchased from Sigma Chemical Company.

Enzyme assays

General esterase activity was determined spectrophotometrically with NA as substrate, by a modification of method developed by Van Asperen (1962). Protein assay of the enzyme source was determined by method of Read and Northcote (1981). Inhibition study was followed by the method of Stephen and Cheldelkin (1971), and of Bigly and Plapp (1965).

The following is the general set-up of a typical experiment: 0.1 ml of substrate solution (5×10^{-4} M NA in 1% acetone and 0.1 M phosphate buffer PH 7.5) was mixed with 1.0 ml of a homogenate containing 1/120 to 1/60 larva equvalent (LE), and was incubated at 27°C for 15 minutes, followed by adding diluted diazoblue lauryl sulfate solution (DBLS) to 3.0 ml. After another 15 minutes, the absorbancy of the mixed aliquot was measured at 600 nm.

In order to characterize the enzyme from the supernatant of the larval homogenate, factors that affected the esterase activity were introduced into this set-up. These factors in cluded pH, incubation temperature, enzyme concentration, substrate concentration, incubation time, protein content of sample solution, and solvent.

Enzyme storage

Enzyme samples were stored under three different temperature conditions: 0°, −20° and −80°C, to test the suitable condition for the enzyme storage. Enzyme solution of one LE per ml was kept under these conditions in a period of time. The sample was assayed for the esterase activity after the storage. Period of storage can be varied from one hour to several months depending on the rate of inactivation.

Subcellular distribution of esterases

For the study of subcellular distribution of larval esterases, the 1,000×g supernatant was further centrifuged at 10,000×g for 15 min, and at 100,000×g for 60 min. Four fractions were collected from the centrifugation: cell debris (1,000×g pellet), mitochondrial fraction (10,000×g pellet), microsomal fraction (100,000×g pellet), and soluble fraction (100,000×g supernatant). An aliquot of each fraction was used for assay accordingly. In this experiment the enzyme source with a concentration up to half larva equivalent per

ml was used, especially for the microsomal, the mitochondrial, and the cell debris fraction.

Calibrated standard curves

Two standard curves (data not shown) were established for calibrating the protein content of the enzyme solution, or for calibrating the product formation of the esterase reaction. In the former curve, the concentration of BSA which was ranged from 5 μg/ml to 70 μg/ml were used as standard concentration. In the later curve, the final concentration of naphthol which was ranged from 10^{-5} M to 95×10^{-4} M, was used for the measurement of hydrolysis of NA.

RESULTS

pH stability

The esterases are most stable under neutral and mildly acidic condition, *i.e.*, pH 7.7–6.5 (Fig. 1). It becomes less stable when the pH is either higher than 7.7, or lower than 6.5. There has very little effect of the buffer pH on the activity when enzyme concentration is low. The effectiveness of the buffer pH to the esterases is detectable when higher enzyme concentration was used. The maxium activity was estimated when pH 7.5 buffer was used for the assay. The activity declined gradually with increasing pH. The non-linear line of the pH versus esterase activity reflected that most of the esterases are of the B-type. Our speculation on the esterase category was also confirmed by the result of inhibition study.

Two concentrations 0.05 M and 0.1 M of the buffer were tested for the influence of buffer concentration to the enzyme activity. Results (data not shown) revealed that either concentration was useable for the assay.

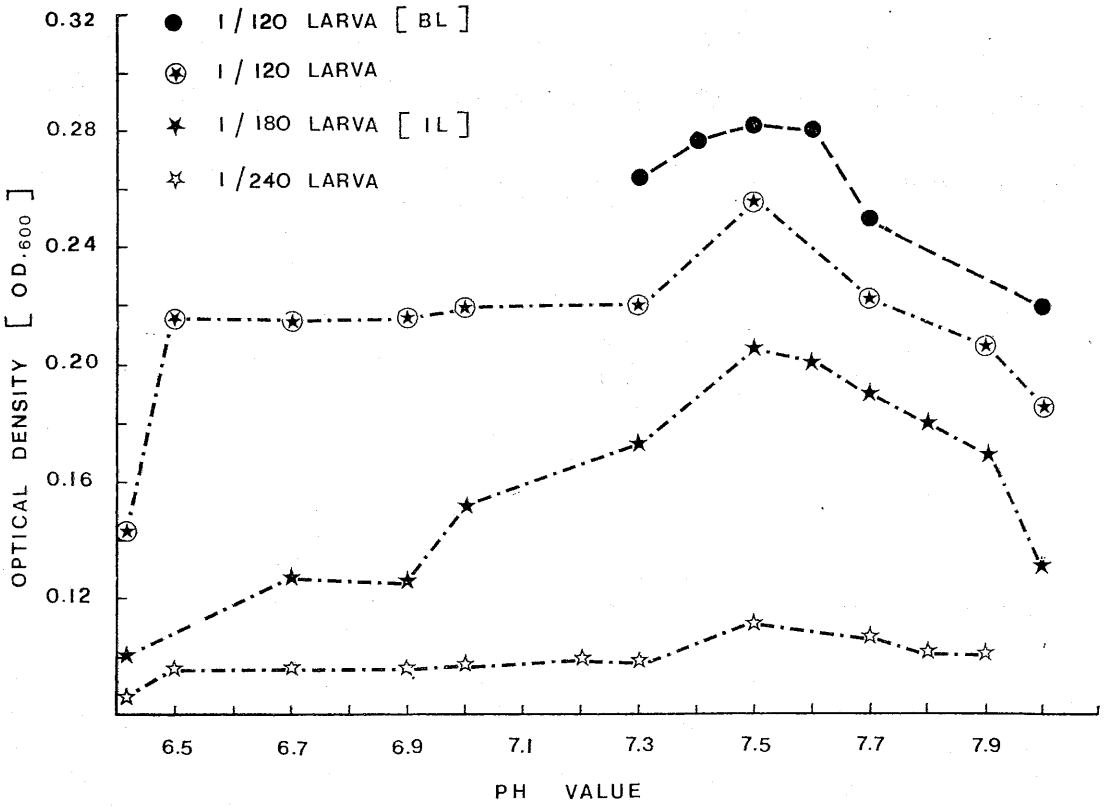

Fig. 1. The influence of pH on esterase activity. The larvae were collected from Bamboo Lake and I-lan. Conditions as stated in Materials and Methods.

Temperature stability

Fig. 2 shows the Arrhenius plot forward and reactions of esterase in phosphate buffer. The straight line of the plot reflected that no heat inactivation of the esterases was detected when the incubation was set under 40°C. Incubation with higher temperature was not tried since we found 27±1°C was the most satisfied and reproducible condition for the assay.

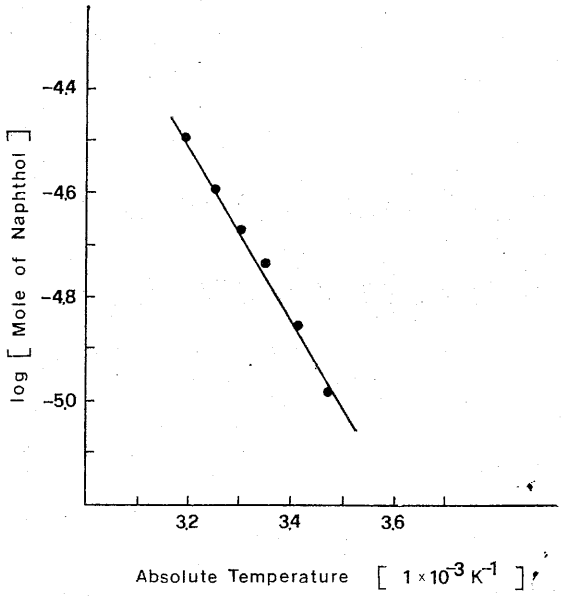

Fig. 2. The influence of temperature on esterase activity. The larvae were collected from Bamboo Lake. Conditions as stated in Materials and Methods. Arrhenius plots to testify the effects of temperature on reaction rates.

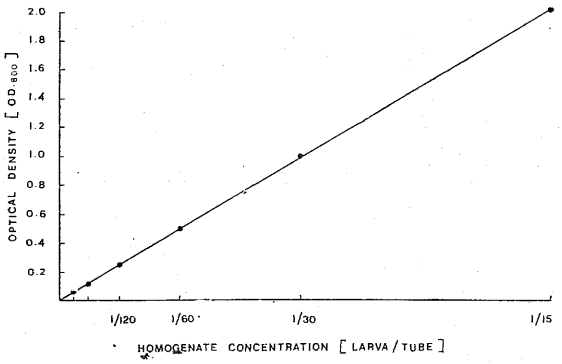

Fig. 3. Activition of larval esterases at different homogenate concentrations. The larvae were collected from Bamboo Lake. Conditons as stated in Materials and Methods.

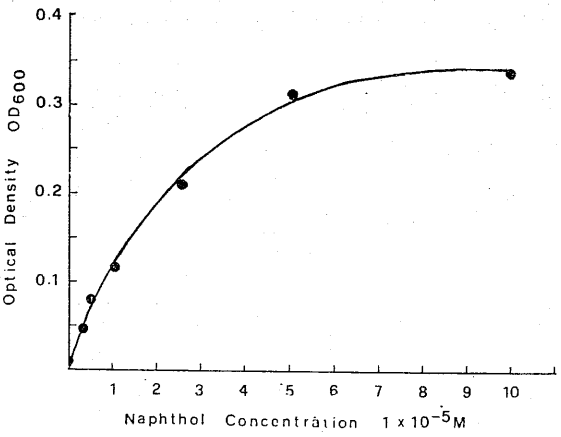

Fig. 4. The effect of 1-naphthylacetate concentration on its hydrolysis. The larvae were collected from Bamboo Lake. Conditions as stated in Materials and Methods.

Concentration of enzyme and substrate

Fig. 3 illustrates that only minute amounts of homogenate enzymes are needed for the estimation of esterase activity and that the enzymes are very efficient in converting the substrate into product even with a sub-optimal substrate concentration. Fig. 4 shows the effect of increasing substrate concentation on enzyme reaction. The relationship is a rectangular hyperbole, showing maximum hydrolysis at 1×10^{-4} M NA. Therefore, 5×10^{-4} M NA is used for all of the esterase assay. Whileas, we use 1.0×10^{-4} M NA, as the maximum substrate concentration for kinetic study. Fig. 5 shows that the Michaelis-Menten transformation of the data resulted in a straight line. From this plot an apparent K_m value of $(3.5\pm0.4)\times10^{-5}$ M, and V_{max} value of $(6.1\pm0.2)\times10^{-6}$ M can be calculated. Although, variation of the K_m and V_{max} values was found due to seasonal flutuation of enzyme activity.

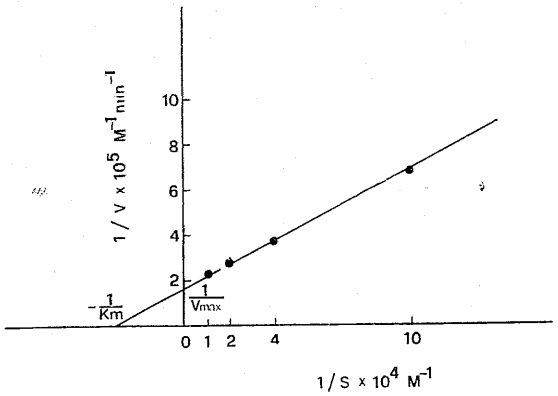

Fig. 5. Lineweaver-Burk plots showing K_m and V_{max} for 1-naphthylacetate hydrolysis by 3/20 larval equivalent, 1,000 ×g supernatant. Conditions as stated in Materials and Methods. Bamboo Lake population.

Incubation time

Under defined conditions the rate of NA hydrolysis is linear with the incubation time during the first 15 min and the reaction is declined somehow during the next 15 min. In the first 15 min incubation the rate of hydrolysis is directly proportional to the amount of the enzyme concentration, which provided that the total hydrolysis does not exceed 12.6% of the amount of the substrate when 5×10^{-4} M NA is used for the survey.

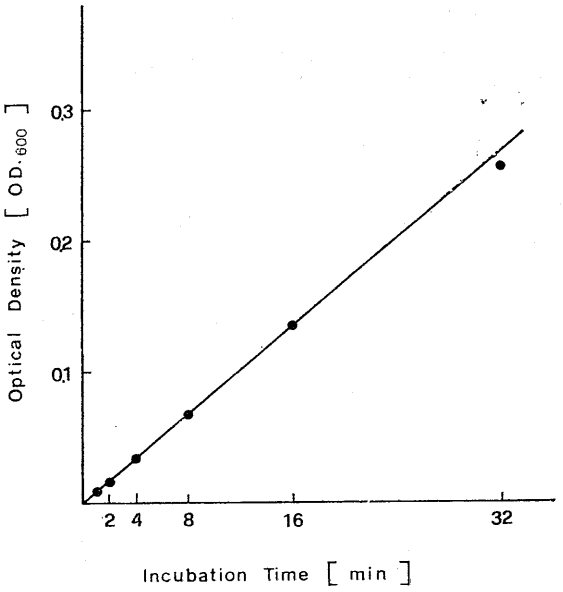

Fig. 6. The influence of reaction time on esterase activity. The larvae were collected from Bamboo Lake. Conditions as stated in Materials and Methods.

Effect of inhibitors and solvent

In the experiments described above, the fractions possibly contained arylesterases and choline esterases, both of which will hydrolyze naphthylacetate. To determine the contribution of these additional esterases in the 1,000 g supernatant, inhibitors; such as eserine, PHMB and paraoxon were used to characterize the esterases of the larval homogenate. Table 1 shows that 10^{-5} M eserine inhibited 50% of the enzyme activity. Comparatively, 10^{-4} M PHMB was found ineffective to the enzymes, indicating that the arylesterase is very minor in the larval esterases. On the other hand, 1×10^{-8} M paraoxon strongly inhibited the enzyme activity indicating that most of the larval esterases are of the B-type, sensitive to organophosphate compounds.

Five to ten percent ethanol solution enhanced the enzyme activity. The activity somehow decreased when high concentration of ethanol was used. The 5% acetone solution was found with no effect to the larval esterases, and neither BSA (Table 1).

Enzyme storage

Table 2 shows that the enzyme activity decreases rapidly at 0°C. The decaying rate of the esterase activity was around 10% or more of the initial activity within the first 5 hours. Low temperature as −20°C was efficient for the esterase of intact insect, but not for that of the homogenated and centrifugated larval preparation. Experiment shows that the enzyme activity of the intact larval preparation increased by the end of the first week of storage. Meanwhile, the esterase activity of the homogenated and centrifugated larval preparation decreased to 60% of the initial. The different decaying rate of the homogenate larval esterases, *e. g.*; fast degradation of the esterase in the first couple days and slow degradation

TABLE 1
The effect of different reagent to the esterase activity of diamond-back moth larvae

Reagent	Concentration	Effect
Eserine	$(1.00\pm0.1)\times10^{-5}$ M	50% inactivation
Paraoxon	$(7.35\pm0.05)\times10^{-12}$ M	50% inactivation
	$(1.91\pm0.2)\times10^{-8}$ M	90% inactivation
PHMB	$(1.0\times10^{-4}$ M)	not detectable
Ethanol	5-10%	enhancement
Acetone	5-10%	not detectable
	15%	inactivation
BSA	5 μg-70 μg/ml	not detectable

Esterase activity of 1,000 g homogenate, 1/20 larval equivalent. Bamboo Lake population.

TABLE 2
The influence of storage temperature to the esterase activity of diamond-back moth larvae

Period of storage		Percentage of enzyme activity†♯†				
		0°C	−20°C		−80°C	
Insect population		GF*	CH‡	BL*	GF*	BL*
Hrs	0	100				
	5	88.6				
Days	0		100	100	100	100
	1		95.9	83.7	103.6	—
	2		115.1	66.8	101.4	—
	3		—	—	101.4	—
	4		120.5	63.8	101.4	—
Weeks	1		116.4	58.2	110.4	—
	2			57.1	110.9	—
	3			47.4	106.6	—
Mts	1				116.8	—
	2				101.0	99.0
	3				96.2	95.1
	4				110	110

* Enzyme stored as homogenate.
‡ Enzyme stored as intact insect.
† Esterase activity of 1,000 g homogenate 1/20 larval equivalent.
♯ Means of triplicates of 2 experiments.

of that during the consequent period, reveals that various categories of esterases were possibly presented in the larval homogenate.

The loss of the esterase activity is arrested when appropriate storage condition is adopted. Esterase preparation stored under −80°C can greatly improve the storage condition. In fact, the homogenated enzyme solution can be kept in active condition as long as we tested, *e. g.*, for four months or more. The mechanism of the enzyme decay and the long lasting storage of the enzymes under such a low temperature was not known.

Subcellular distribution of esterases

Table 3 shows that 68% of the total esterase activity was present in the 100,000×g supernatant, the soluble protein fraction. Whiles, esterases of all membraneous fraction contributed only 26% of the total activity. Enzyme activity recovered from these four fractions was counted as 94.4% of that of the 1,000×g supernatant of the larval homogenate. The table also shows that when the enzyme specific activity was taken into account, the membraneous esterase activity is supperior to the soluble esterase. Another interesting aspect of the result is that when kinetic parameters; K_m and V_{max} were used to evaluate the esterase activity, we find that both mitochondrial and microsomal esterases is more efficient in spliting the substrate than the soluble esterases. This is the case with special interesting because microsomal esterases obviously played an important role on the ester hydrolysis as a defensive mechanism, to ester-containing pesticides, of the insect.

TABLE 3
The subcellular distribution of the esterase activity of diamond-back moth larvae*

Fraction	Enzyme activity (mole^{-5}/0.5 LE/15 min)	Protein content (μg protein/0.5 LE)	Specific enzyme activity‡ (mole^{-5} of product/ mg protein/ 15 min)
1,000 g supernatant of homogenate	18.1	675	26.8
Cell debris and nuclei	1.23	70.4	17.5
Mitochondrial fraction	1.58	62.0	25.5
Soluble fraction	12.4	512.7	24.2
Microsomal fraction	1.87	61.3	30.4

* Bamboo Lake poulation, last instar larval.
‡ Mean of two experiments with triplicates each.

DISCUSSION

This is a preliminary report of *in vitro* study on the larval esterases of diamond-back moth. The results presented here is to describe the kinetic properties of the enzymic hydrolysis of the naphthylacetate, quantitative data on the rate of substrate hydrolysis under defined conditions, and the effectiveness of the temperature for enzyme storage. Moreover, esterase activity derived from different subcellular fractions were also explored.

In its general characteristic the category of the larval esterases of the diamond-back moth resembled that of other insect species studied (Asperen, 1962, Katzonellembogen and Kafatos, 1971). The optimal condition for the non-specific esterases of the larva was determined. One tenth molar phosphate buffer at pH 7.5 in 27°C for 15 min gave the maximum activity for esterase reaction. The kinetic constant, K_m, of the enzyme was measured 3.5×10^{-5} M for the substrate (Fig. 5). The esterases were very susceptible to paraoxon, but were resistant to PHMB (Table 1). The hydrolytic reaction of these enzymes, unlike the cholinesterase, was found less pH-dependent (Fig. 1). These evidences reflected that the larval esterases were of B-type.

Study on subcellular distribution of esterase revealed that the majority of the enzyme (68%) was presented in the soluble fraction of the homogenate, and the minority (26%), in membranous systems (Table 3). This measurement was based on activity per larval equivalent. When we took the enzyme specific activity into account we found that microsomal esterases was the most efficient isozymes to

split the substrate. Kinetic study on esterase to NA revealed that the affinity of NA to microsomal esterases is around 1.5 fold as that to soluble esterases, and the turnover rate of the substrate by these enzymes was around 3.0 folds between the two different fractions, favoring to the microsomal esterases (data not shown). In other words, the microsomal esterases probably played an important role in pesticide degradation other than the soluble esterases. The population-dependent variation of soluble esterases of diamond-back moth larvae were first detected by Sun *et al.* (1978). They did not find a positive interrelation between the pesticide-resistance and the number of the esterase isozymes. With all the above mentioned evidences it leaded to an assumption that the microsomal esterase might play a major role on the pesticide-detoxication in the diamond-back moth. Further investigation on this subject will be continued in this laboratory.

Another interesting aspect of this study is the factor influencing the enzymatic catalysis of the esterases during storage. The result (Table 2) shows that the esterase of frozen intact insects is as active either as those of freshly prepared enzyme or as those of freshly prepared and deep frozen enzymes (−80°C). It is obvious that there presented endogenous interfering reagent or inhibitors of the esterase in the enzyme solution of the homogenated larvae. Similar case was found in other insect (Lien and Casida, 1974). In this study we find that the stored enzyme preparation can be activitated to more than ten percent of the initial. This increase of esterase activity was found during the first seven days of the storage. Schonbrod and Terriere (1966) indicated that the housefly enzyme increased its activity during storage. They postulated that the increase in activity was partially due to the changes in the protein structure of the enzyme. Similar assumption can be applied to the result of our observation. To improve the storage condition for the enzyme preparation is another subject worthy of further investigation.

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小菜蛾 (*Plutella xylostella* L.) 之酯酶
I. 幼蟲酯酶之酵素特性

馬堪津　莊美玲

本報告的主旨在探討小菜蛾 (*Plutella xylostella* L.) 幼蟲酯酶水解醋酸萘酯所須最適宜的反應條件，及酶活性之大小。經實驗證明，在攝氏二十七度、酸鹼度爲七點五、十分之一摩爾濃度之磷酸鉀緩衡液中酯酶之作用速率最大。

以抑制劑處理酯酶得知絕大部份酶是羰酯酶。反應液中之有機溶劑能影響生成物之產生，而蛋白質之含量則不起影響作用。用攝氏零下八十度處理酯酶可常保其活性，若換置在零下二十度或更高溫度，則酶之活性隨貯存的時間延長而遞減。

受質與酯酶之親和力測知爲 $(3.5\pm0.4)\times10^{-5}$ M，而受質分解之更新率爲 $(6.1\pm0.2)\times10^{-6}$ M。超速離心分離出之水溶性酯酶其活性約佔全部酯酶活性的 68%，其他膜質酯酶 26%。若以酶特殊活性來估計，則後者較前者爲强。文中也討論到這些酶與小菜蛾幼蟲解毒作用之關連性。