中華農業研究 (Jour. Agric. Res. China) 49(4): 89～98 (2000)

Inhibitory Characteristics of Methamidophos on House fly Head Acetylcholinesterase[1]

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Abstract: The acetylcholinesterase (AChE) purified from housefly (*Musca domestica* L.) head of a TARI's colony has been tested for its sensitivity to methamidophos, a phosphoramidate that is highly water-soluble but with low sensitivity to AChE. The reaction of methamidophos to AChE was analyzed *in vitro* in several insecticide concentrations (0.3, 0.6, 1.2, 2.3 and 4.6ppm) at different incubation times. In general, the activity of AChE remains unchanged in room temperature for 30 min, and all the results confirm that action of methamidophos to AChE is slow but stable. By increasing the incubation time, strong inhibition can be resulted from low concentration of methamidophos. For example, 0.6ppm results in 37% inhibition in 30 min, while 4.6ppm only cause 36% inhibition in the 5 min treatment i.e., the 25 min difference in incubation time can cause 8-fold increase in insecticide sensitivity. By utilizing this character, the housefly head AChE can be used to detect the methamidophos residue in plant material simply by increasing the incubation time. In the actual measurement, almost 100% methamidophos residue was recovered by phosphate buffer extraction instead of methanol/ethanol extraction, and the sensitivity of AChE to methamidophos residue can extend to 2-4ppm level when testing the marketable size cabbage. We conclude that by modifying the testing procedures, the housefly head AChE is feasible for monitoring methamidophos residue on plant material.

Key words: Housefly head acetylcholinesterase, Methamidophos, Enzyme stability, Incubation time, Recovery.

INTRODUCTION

It is well known that the target site of organophosphorus insecticides is the acetylcholinesterase (AChE) of animal neural system[9]. The organophosphorus insecticide constitutes a large group of chemicals. The most common one of this group is the organic phosphorothionates, which have to be activated into corresponding -oxon analogs to become effective[5]. Usually, the activated insecticides cause *in vitro* inhibition on AChE is at 10^{-7} ~10^{-9} molar/liter. However, methamidophos, acephate and several other insecticides constitute another effective organophosphorus group i.e., phosphoramidates, and have different *in vitro* AChE inhibition

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characteristics. Theoretically, compound such as methamidophos with $ki = 9.2 \times 10^{-2} M^{-1} min^{-1}$ and $I_{50} = 3.9 \times 10^{-5}$ M versus fly head acetylcholinesterase was not considered as an effective insecticide. Since it had no obvious leaving group, and nothing to bind the anionic site[7] and the molecule is too small to reach the anionic site (5 Å from the esteratic site) in any dimension, which generates great interest in research on their modes of action.

At first, a possible extremely effective impurity in methamidophos was suspected. Magee had recrystalized methamidophos 10 times, and Fukuto[4] still obtained same AChE inhibition ability and ruled out the possibility of impurity[7]. The possibility of *in vivo* activation was also tested, and Khasawinah *et al.*[6] had found no degradation of methamidophos in eight hr in both resistant and susceptible strain houseflies. By incubating 6 hr with cockroach gut and mouse liver slices, the breakdown product of methamidophos was not active on AChE. Magee and McCaskey[8] studied the oxygen analogs of methamidophos and demonstrated that it has no toxicity, and the *in vitro* activation of methamidophos could not be proved. Methamidophos is an insecticide with simple structure (mol.wt. 141) and high deliquescence. This molecule showed superior hydrolytic stability and had no degradation for eight hr in housefly. Therefore, it is speculated that the slow but cumulative *in vivo* inhibition of AChE by unchanged methamidophos caused the poison[7].

In this study, the housefly head acetylcholinesterase (FH AChE) purified from a housefly colony in Taiwan Agricultural Research Institute (TARI) was investigated for its characteristics of methamidophos inhibition. Direct evidences of Magee's hypothesis of slow and stable methamidophos action on AChE will be sought, and the possible usage of AChE in methamidophos residue detection is also studied.

MATERIALS AND METHODS

I. Enzyme, insecticide and other materials used:

AChE is purified from the head of housefly, *Musca domestica* L, reared in TARI[1], and the enzyme for inhibitory studies is the lyophilized powder stored under 0 ℃. The AChE at 0.40 Δ Abs/min/20μl was used for all the studies, and the Ellman's test[2] was adopted for AChE activity determination. Other reagents used for the assay are DTNB (5,5-dithio-bis-2-nitrobenzoic acid, purchased from Merck), Na_2HPO_4 (from Merck), $N_aH_2PO_4$ (from Merck) and ATCI (Acetylthiocholine iodine, purchased from Sigma).

Ellman's test:

(a)

$$(CH_3)_3\overset{\oplus}{N}CH_2CH_2SCOCH_3 + H_20 \xrightarrow{AChE} CH_3COOH + (CH_3)_3\overset{\oplus}{N}CH_2CH_2SH$$

Acetylthiocholine — acetic acid — thiocholine

(b)

$$(CH_3)_3\overset{\oplus}{N}CH_2CH_2SH + O_2N\text{-}C_6H_3(COO^{\ominus})\text{-}S\text{-}S\text{-}C_6H_3(COO^{\ominus})\text{-}NO_2$$

Thiocholine — DTNB

$$\longrightarrow (CH_3)_3NCH_2CH_2SS\text{-}C_6H_3(COO^{\ominus})\text{-}NO_2 + O_2N\text{-}C_6H_3(COO^{\ominus})\text{-}S$$

2-nitrobenzoate-5-mercaptothiocholine — 5-thio-2-nitrobenzoate

Methamidophos 50%EC was purchased as commercial product, and diluted with distilled water to 1000ppm as stock solution, then serial dilutions were made form the stock solution. Usually the testing concentrations for methamidophos were 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9 and 2.0ppm. The inert ingredient of the emulsifier concentrate formulation did not interfere with the AChE activity.

II. The leaf dipping treatment:

Greenhouse-grown insecticide-free cabbage seedling, marketable size common cabbage and Triton X-100 (Sigma) were used for leaf dipping test. Cabbage leaves were cut into leaf discs (2.4cm in diameter), and dipped into methamidophos solution with 200ppm Triton X-100 and leave dry in room temperature.

III. Instruments used:

The absorbance change of the reaction was assayed on spectrophotometer (Shimadzu UV-1201V) and ELISA reader (Model: SpectraMAX340, Molecular Devices) with kinetic mode.

IV. Testing procedures and solutions used for methamidophos inhibition tests:

i. Spectrophotometric method: concentrations and reagents used were AChE (3mg/ml distilled water, ca. 0.40 ΔAbs/min/20μl), DTNB (19.8mg/ml), ATCI (21.6mg/ml distilled water) and 0.1M PBS (pH8.0, also used as extraction solution).

A. 0.5ml PBS, 20μl (or 50μl) insecticide solution and 20μl AChE were incubated first, then added 2.5ml PBS, 20μl ATCI and 100μl DTNB in the above mixture to start the reaction.

B. 4ml PBS, 4 leaf discs and 40μl AChE were incubated first, then transferred 3ml incubated mixture into a cuvette, and added 20μl ATCI and 100μl DTNB to start the reaction.

ii. ELISA reader method: 0.1M PBS (pH8.0), AChE solution (3mg/ml distilled water) and diluted DTNB and ATCI mixture (0.4 mg of DTNB + 0.4mg of ATCI /ml distilled water) were used. Four milliliter PBS , 4 leaf discs and 40μl AChE were incubated first, and 100μl of the above mixture was transferred into a reading well of microplate, then added 50μl mixture of ATCI and DTNB for assay.

V. Investigated items:

i. AChE stability test: To examine whether the FH AChE is stable enough to conduct the methamidophos incubation study.

A. AChE (20μl) was incubated in 3ml PBS in room temperature for 3, 5, 10, 15, 20 and 30 min, and then adding 100μl DTNB and 20μl ATCI according to the spectrophotometric method to start the reaction.

B. AChE was incubated in 4ml PBS with 4 pieces of clean cabbage leaf discs and incubated for 3, 5, 10, 15, 20 and 30 min in room temperature. Then transferred 3ml of the mixture into a cuvette, and the reaction was started by adding DTNB and ATCI according to the spectrophotometric method.

The absorbance change per minute was recorded, and the AChE activity (ΔAbs /min) of different treatments were compared.

ii. The effect of methamidophos reaction time on AChE: In this study, the reaction time of methamidophos on AChE is investigated. Twenty microliter AChE and 20μl of methamidophos solution were incubated in 0.5ml PBS for 5, 10, 15, 20 and 30min. After incubation, start the reaction according to the spectrophotometric method used in IV-i-A. All the tests were compared to the control test without methamidophos. By adjusting the volume of methamidophos solution to 50μl, the second set of test was performed for comparison.

iii. The AChE detectability of methamidophos on plant: In this test, the possible usage of AChE for

methamidophos residue detection is explored.

A. Forty microliter AChE was incubated with 4 pieces of methamidophos-treated leaf discs in 4ml PBS for 5, 10, 15, 20 and 30 min. After incubation, 3ml mixture was transferred into the cuvette, and the reaction was started according to the spectrophotometric method described in IV-i-B.

B. Same experiment was conducted by ELISA reader with kinetic mode for massive sampling technique according to IV-ii method.

The results of both tests were compared for repeatability and adjustment.

RESULTS AND DISCUSSION

AChE stability study:

In the buffer only treatment, the FH AChE activity was 0.4 ΔAbs/min and remained unchanged for 30 min in room temperature. When incubated with 4 leaf discs, more enzyme (40μl) was used to compensate the possible absorption of leaf surface, and the AChE activity stay at 0.53 ΔAbs/min for 30 min (Table 1). Both results indicated that the FH AChE of TARI is stable enough to perform the methamidophos incubation test up to 30 min in room temperature without any problem of activity degradation.

Table 1. AChE stability in room temperature

Incubation solution	Assay solution	ΔAbs/min in different incubation time ±(S.E.)					
		3 min	5 min	10 min	15 min	20 min	30 min
3ml PBS buffer +20μl AChE	3.02ml mixture +100μl DTNB +20μl ATCI	0.40 (0.01)[z]	0.39 (0.01)	0.40 (0.01)	0.39 (0.01)	0.41 (0.01)	0.41 (0.01)
4ml PBS buffer +0.4g leaf discs +40μl AChE	3ml mixture +100μl DTNB +20μl ATCI	0.54 (0.01)	0.51 (0.01)	0.53 (0.01)	0.55 (0.01)	0.53 (0.01)	0.54 (0.01)

[z] The difference is not significant.

The effect of methamidophos reaction time on AChE:

The inhibitory effect of various concentrations of methamidophos at different reaction time was measured (Figures 1). In this test, inhibition of AChE beyond 35%, the current warning level used in toxicity test, is considered to be significant. Incubation of AChE with methamidophos for 5 min does not cause significant inhibition at concentration of 1ppm or below, and a 36% inhibition was observed at 4.6ppm treatment. Increasing the incubation time lead to the improvement of the inhibitory effect of methamidophos to FH AChE. For example, 0.6ppm methamidophos resulted in 37% inhibition in the 30min treatment, an 8-fold increase (4.6ppm/0.6ppm = 7.7) in sensitivity. The dilution of methamidophos in the first test is 27-fold (20μl in 540μl incubation mixture), and is 11.4-fold in the second test (Fig. 2) (50μl in 570μl incubation mixture). However, the inhibitory responses of AChE in relation to methamidophos dosage were similar in both tests.

The data of Figures 1 and 2 were analyzed by probit[3] to establish the regression between AChE inhibition and methamidophos dosage. The estimated correlation was presented in Table 2. For 30 min treatment, the sensitivity of AChE to methamidophos in the first test is tabulated on I_{75}, I_{50}, I_{45}, I_{35}, and the corresponding dosages are 2.23, 0.91, 0.76 and 0.54ppm, respectively. In the second test, the correspondent dosages were 2.50, 1.19, 1.04 and 0.75ppm, respectively, and they are not significantly different from the first test. When

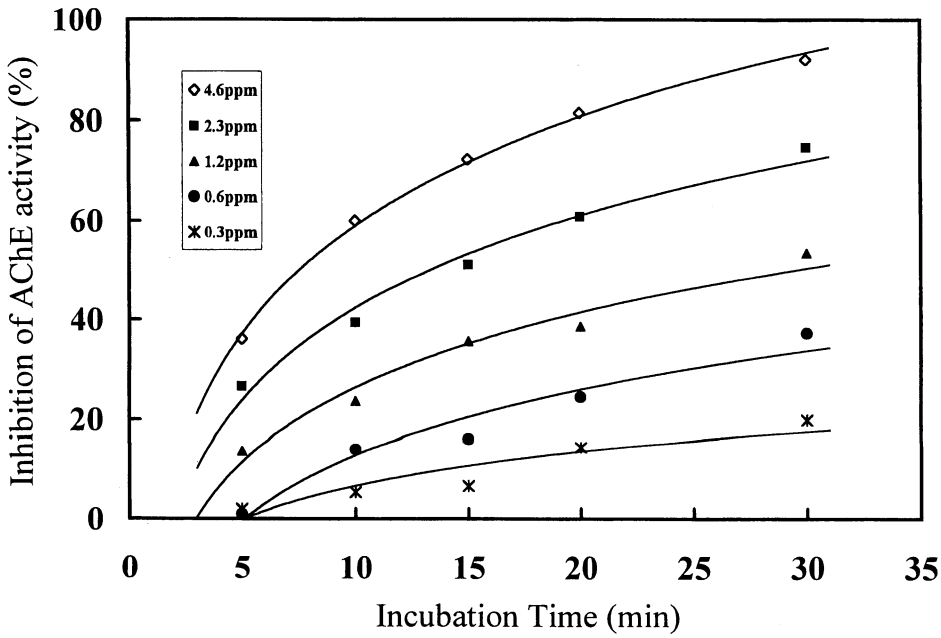

Fig. 1. Observed AChE inhibition by different methamidophos concentrations at different incubation times.
Note: (1) 0.5ml PBS + 20μl AChE + 20μl methamidophos solution for incubation (D.F. = 27X).
(2) After incubation, added 100μl DTNB and 20μl ATCI to assay the AChE activity.
(3) The treated AChE activity was compared with control for inhibition.

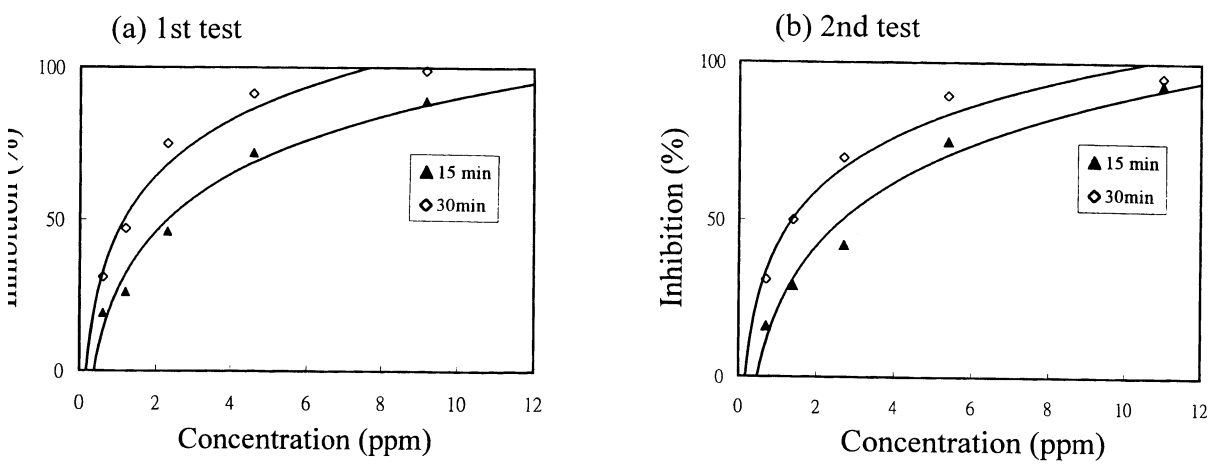

Fig. 2. Observed AChE inhibition of methamidophos at two dilution tests.
(a) 1st test: 0.5ml PBS + 20ul methamidophos + 20ul AChE (D.F.=27X)
(b) 2nd test: 0.5ml PBS + 50ul methamidophos + 20ul AChE (D.F.=11.4X)

incubated 15min, two tests also produced similar results. Both sets of test results were then averaged in the mean values of corresponding dosages in Table 2.

Table 2. Estimated concentration of methamidophos to produce the AChE inhibition at different incubation times

% inhibition	15 min incubation			30 min incubation		
	D.F. = 27X	D.F. = 11.4X	Ave.	D.F. = 27X	D.F. = 11.4X	Ave.
25	0.93ppm	1.12ppm	1.03ppm	0.37ppm	0.57ppm	0.47ppm
35	1.36	1.60	1.48	0.54	0.78	0.66
45	1.90	2.20	2.05	0.76	1.04	0.90
50	2.24	2.55	2.40	0.91	1.19	1.05
75	5.38	5.77	5.58	2.23	2.50	2.37
90	11.87	12.04	11.96	5.03	4.88	4.96

Observations of Figures 1 and 2 provided direct evidence that the inhibitory effect of methamidophos on FH AChE is slow, stable, and cumulative. If the methamidophos were not easily hydrolyze *in vivo*, its slow poisoning to AChE in nerve system will become significant and eventually cause biochemical blocking of the nerve function. Although the action of methamidophos on AChE is slow and stable, it is dosage-dependent. In Figure 3, the % AChE inhibition/min was calculated at different point of incubation time. At 4.6ppm, a complete inhibitory AChE is estimated at 40-50 min and the regression pattern (line II) is different from that of 1.2ppm (line I), which induced much slower AChE inhibition progress rate.

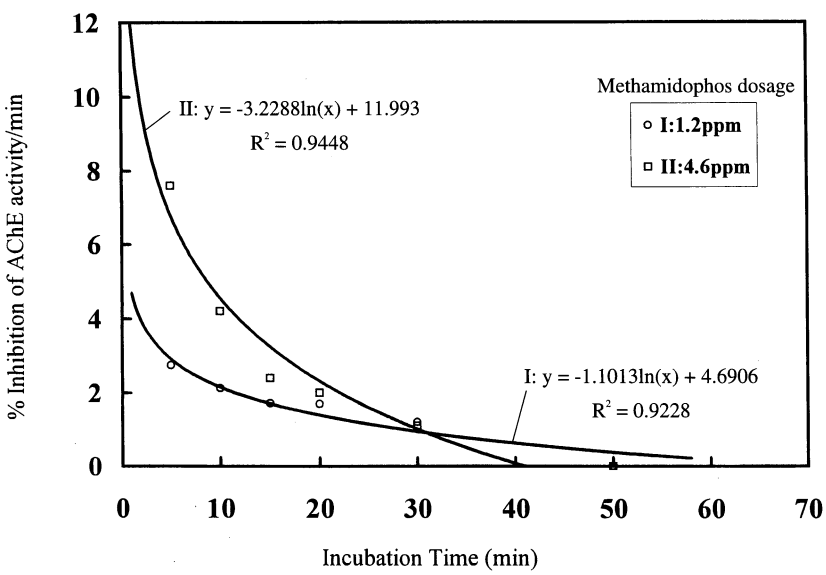

Fig. 3. Inhibitory effect of methamidophos to AChE in relation to the incubation time at two dosages.

The comparison in Figure 3 reflects a difference of *in vitro* test from *in vivo* poison. Although the methamidophos poisoning is slow and stable at the beginning (at low concentration or line II), if the animals

continue to absorb methamidophos, increased dosage in the body fluid will change the progress of AChE inhibition from regression line I to regression line II. In other word, more rapid. While in the *in vitro* study, only the fixed dosage response of AChE can be observed.

The AChE detectability of methamidophos on plants:

In the third part of this study, we found the methamidophos residue on plant material can be detected by FH AChE down to the ppm level. The results are presented in Table 3. In the dipping test, the cabbage leaf dipped with 31.25ppm or higher concentration of methamidophos showed very consistent AChE inhibition, hence we consider the sensitivity threshold is at 31.25ppm, and following discussion is mostly based on this dosage. A parallel test on ELISA reader provides the same result (Table 3, Part II).

Table 3. Comparison of methamidophos inhibition on AChE at different incubation times

Concentration of methamidophos for leaf dipping, ppm	% AChE inhibition				
	5 min	10 min	15 min	20 min	30 min
(Test by Spectrophotometer)					
250ppm	100	100	100	100	100
125ppm	56	74	95	97	98
62.5ppm	52	64	61	82	83
31.25ppm	20	43	45	33	43
15.63ppm	0	6	0	0	0
(Test by ELISA reader)					
250ppm	91	92	94	95	97
125ppm	44	56	69	90	91
62.5ppm	16	51	58	66	69
31.25ppm	20	39	44	46	39
15.63ppm	0	5	0	0	3

When the cabbage leaf was dipped in 31.25ppm and assayed in the 4-leaf discs testing procedure, the presence of methamidophos can be detected by FH AChE inhibition at 35% or more (Table3, Part I). Equivalent study on ELISA reader provided the same result and presented in part II of the same Table. The cabbage seedling leaf is rather thin in thickness compared with the marketable size common cabbage, and the weight of 4 leaf discs is 0.4gram, which can pick up 0.096gram of 31.25ppm solution or 3.0μg methamidophos and resulted in 7.5ppm residue (Table 4) and 45% AChE inhibition. The 45% AChE inhibition is corresponding to the 0.9ppm dosage response in Table 2. In 4.04ml reaction solution, 0.9ppm equivalent to 3.6μg and this figure is close to the estimated uptake of 3μg in leaf dipping treatment or an almost 100% recovery was obtained in residue analysis. The high recovery of methamidophos in buffer solution fits the physical character of high water solubility of methamidophos.

Table 4. Amount of methamidophos deposition and AChE inhibition by leaf dipping treatment at 31.25ppm

Cabbage size	Weight of leaf disc, g		Weight of methamidophos solution, g	AChE inhibition
	Before dipping	After dipping		
Seedling	0.1005 ± 0.0167^{Z}	0.1244 ± 0.0136	0.0239 ± 0.0053	ca. 45%[X]
Marketable	0.2558 ± 0.0998	0.2895 ± 0.0770	0.0337 ± 0.0094	>45%

[Z] n = 20.

[X] 45% inhibition on AChE activity is considered as the detection threshold.

<Estimated recovery of methamidophos by PBS extraction>

(i) Cabbage seedling: 31.25μg/g x 0.0239g x 4 = 3μg

3μg/(0.1005 x 4)g = 7.43ppm.....methamidophos residue

(ii) Marketable size cabbage:

31.25μg/g x 0.0337g x 4 = 4.2μg

4.2μg/(0.2558g x 4) = 4.2ppm.....methamidophos residue

I_{45} in Table 2 is 0.9ppm, and 4.04ml x 0.9ppm = 3.6μg

For the marketable common cabbage, the leaf is much thicker than that of seedlings and its weight is 0.256gram per leaf disc (Table 4). In dipping treatment, 31.25ppm methamidophos solution was absorbed in a unit of 0.034 gram/disc. For 4 leaf discs, 4.25μg methamidophos was deposited on 1.024g of plant material, of 4.15ppm residue, and higher than 45% AChE inhibition was recorded, hence the FH AChE detection sensitivity at 35% inhibition for methamidophos residue in plant is estimated at 2-4ppm.

In the ELISA reader test, the results completely match the single AChE test on spectrophotometer (Part II of Table 4).

CONCLUSION

Chiu *et al.*[1] reported that FH AChE was weakly inhibited when incubated with methamidophos for 3 min. The FH AChE of TARI was used in this study since it is stable in room temperature and can tolerate long incubation time. Because the action of methamidophos on AChE is slow, by increasing the incubation time of methamidophos and FH AChE, increased inhibitory effect is expected. The purpose of measuring methamidophos sensitivity to FH AChE is to explore the possibility of utilizing FH AChE to detect methamidophos residue on plant to the ppm level.

In this study, we have confirmed that the slow and stable inhibitory effect of methamidophos to AChE. By increasing the incubating time, the AChE detectability of methamidophos can be improved. The modified test is fast and sensitive enough to fit the purpose of developing the quick bioassay in methamidophos residue detection. We also found the extraction of methamidophos by aqueous PBS is satisfactory, which might due to the high water solubility of methamidophos, and almost complete recovery of methamidophos from plant material is obtained. The sensitivity of FH AChE in detecting methamidophos residue on plant materials can be measured at the concentration of 4ppm or below, which makes FH AChE a very useful tool in monitoring the methamidophos residue.

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達馬松對家蠅乙醯膽鹼酯酶抑制特質之研究[1]

鄭允、高靜華、黃毓斌、曾佳琳[2]

摘要

早期曾利用農業試驗所飼養家蠅所純化之乙醯膽鹼酯酶，測試其對達馬松作用3分鐘之敏感度，以25%抑制率爲基準時之敏感度甚低。由於達馬松具有高水溶性以及作用於乙醯膽鹼酯酶時反應較慢之特性，本研究乃著力於修正以乙醯膽鹼酯酶檢測達馬松之方法，期能提高兩者間之敏感度。因乙醯膽鹼酯酶可在室溫下反應30分鐘，而其活性仍保持不變，故與達馬松作用時經由不同反應時間(3~30分鐘)及不同濃度(0.3-4.6ppm之間)進行比較測試。結果顯示，作用5分鐘時，需4ppm達馬松才可達到36%抑制，但反應時間增長爲30分鐘時，僅須0.6ppm達馬松即可達37%之抑制，檢測敏感度可提高近8倍。此外，檢測達馬松在葉片上殘留時，無需使用甲醇或乙醇萃取，改爲直接以緩衝液萃取，結果甚爲理想。經由抑制率之估算，緩衝液之萃取回收率幾達100%，可檢測之達馬松殘留量以植物體重量估算，約爲2-4ppm。此一敏感度與容許量之範圍已甚爲接近，而使乙醯膽鹼酯酶用於檢測達馬松殘毒之應用性大幅提昇。

關鍵詞：家蠅乙醯膽鹼酯酶、達馬松、酵素穩定性、回收率、敏感度。

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