Earthworm's Acetylcholinesterase as Biomarker to Monitor the Effects of Pesticides

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ABSTRACT

The effect of three insecticides; methomyl (carbamate), dimethoate and malathion (organophosphates) and one cupper-containing fungicide (Cupper hydroxide) was evaluated *in vivo* on acetylcolinesterease (AChE) isolated from earthworm *Lumbricus terrestirs* to achieve a better understanding of AChE responses to agrochemicals in *L. terrestirs*. Three values of median lethal concentrations of the four compounds were determined from the acute toxicity (0.1, 0.5 of LC_{50} and the LC_{50}). The three insecticides at the three levels of LC_{50} inhibited AChE activity and the inhibition was dose dependent. Methomyl at LC_{50} level had the highest AChE inhibition (98.9%) after 7 days of exposure to contaminated soil. Cupper hydroxide which contains cupper element showed induction of AChE activity at the three levels of LC_{50}s. The maximal induction was at 0.5 of LC_{50} (27.7% followed with 0.1 (20.3%) and the value of LC_{50} (6.8%) compared with control.

Our results further support the use of AChE as an indicator of pesticide contamination, to be included in a battery of biomarkers for monitoring soil toxicity.

INTRODUCTION

Earthworm constitute 80% of the soil invertebrate biomass in most terrestrial ecosystems of the world (Lee, 1985). They are relatively large, immobile and most easily quantified components of soil biota. They preserve and contribute to the overall productivity of soil ecosystem by maintaining soil structure and regulating the turn over of organic matter through their feeding, casting and burrowing (Dash, 1978; Lee, 1985; Parmelee *et al*., 1990). Earthworms also form one of the principal source of animal proteins for many predators and occupy a major compartment in the chemical element cycles (Ferriere *et al.*, 1981). Due to their relatively large size, limited rapidity in soil displacement, slow recolonization and beneficial role in agro ecosystems, earthworms are used as an indicator species for monitoring the impact pollutants, changing in soil structure and agricultural practices (Haque and Ebing, 1983; Heimbach, 1985; Panda and Sahu, 1999; Paoletti, 1999; Ping *et al*; 1999).

Hundreds of manufactured pesticides of different chemical composition are currently used through out world to protect crops against pests. Small amount of applied pesticides reach the target and the rest affect the non-target organisms (Pimental and Levitan , 1986). For instance, organophosphate and carbamate compounds are generally short-lived in the environment and once ingested or otherwise acquired by an organism, they are rapidly metabolized or execreted (Panda and Sahu, 1997). Organophosphate and carbamate insecticides as neurotoxic agents are known to cause acute toxic effect in earthworm (Scott-Fordsmand and Weeks, 2000; Rao and Kavitha, 2004). The site of action of these insecticides is acetylcholinesterase enzyme (AChE) which hydrolyze acetylcholine in the invertebrate nervous system (Corbett, 1974).

Moreover, cupper – containing sprays have been used to control fungal diseases in fruits and vegetable crops (Merry *et al*., 1983). Cupper is the essential element and required by all organisms. However, elevated concentrations of cupper are toxic and when found in soil it may lead to a range of effects including reduced biological activity and subsequent loss of fertility (Dumestre *et al*., 1999).

Poisoning of the nervous system is perhaps the quickest and most effective method of chemically upsetting regular body function (Hoar, 1991). That is why AChE activity is used as a reliable parameter for assessing the poisoning due to pesticides and heavy metals (Reddy and Venugopal, 1993; Sharma *et al.*, 1993; Devi and Fingerman, 1995; Dembele *et al.*, 1999). Few studies were done on the effect of pesticides on kinetic properties of earthworm's AChE, therefore, the objective of the current study was to evaluate the toxicity of some pesticides on earthworm *Lumbricus terrestirs in vivo*. The effect of lethal and sublethal concentrations on the activity of earthworm's AChE was also studied.

MATERIALS AND METHODS

Animals

The earthworm (*Lumbricus terrestirs*) was collected from the Agriculture Research Center garden, Sabahia, Alexandria. They were carefully brought to the laboratory along with the moist soil within an hour. The worms were acclimatized at the lab conditions (at room temperature and 12 hr light/12 hr dark) in the artificial soil (using an evently blended dry weight mixture of 20% kaolin clay soil, 70% silica sand, 10% sphagnum peat and 0.3% calcium carbonate, according to the

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Organization for Economic Cooperation and Development; OECD guideline 207 (OECD 1984) before testing.

Pesticides

a) Organophosphate insecticides:

1) Malathion (Camethion® 57% EC)

It was obtained from the Egyptian Center for Agriculture Services.

2) Dimethoate (Camethoate® 40% EC).

It was obtained from Trading Company for Agriculture Requirements.

b) Carbamate insecticide

Methomy (Methocam® 90% SP)

It was obtained from Trading Company for Agriculture Requirements

c) Cupper-containing fungicide:

Cupper hydroxide (Dakocide® 83.4% WP)

It was obtained from Delta Company for Agriculture Chemicals.

Reagents

All reagents used in the present study were of analytical grade. Acetylthiocholine iodide (ATChI) and 5,5 dithiobis (2-nitrobenzoic acid (DTNB) were purchased from Sigma- Aldrich Chemical Company.

Determination of median lethal concentration (LC_{50}):-

Toxicity experiments were conducted by artificial soil test for 7-days exposure (OECD, 1984). Different concentrations of the tested pesticides were homogeneously mixed with artificial soil. Control was also run in parallel using water alone. Each concentration was replicated three times and placed in plastic containers covered with perforated cloth. Each replicate contain 10 earthworms of approximately equal length (9.52 $\pm$ 0.25 cm) and weight (0.27 $\pm$ 0.039 g). Mortality percentages were recorded after 7 days of exposure and LC_{50} values were calculated according to Finney (1971).

Enzyme source preparation:

One gram of anterior adult worm (from segment 1-7) was homogenized in 5 ml of 0.1 M phosphate buffer (pH 7.4) using polytron for 1 min. The homogenate was centrifuged at 5000 rpm for 30 min. at 4 ^{o}C. using Beckman GS-6R centrifuge rotor (GA-10). The supernatant was used as an enzyme source.

Protein assay:

The protein content was carried out according to the method described by Lowery *et al*., (1951), using bovine serum albumin (BSA) as standard.

Cholinesterase activity assay:

The spectrophoyometric method of Ellman *et al*., (1961) was used, with acetyl thiocholine iodide (ATChI) as a substrate. In a typical assay 50 µl of the enzyme source was added to 3 ml of a 1:1 mixture of 2 mM substrate solution and 2 mM dithiodinitrobenzoic (DTNB). The final concentration of substrate and DTNB in the assay mixture was 1 mM. The changes in absorption at 412 nm was monitored on spectrophotometer Spectronic 601. An assay mixture without enzyme was used as the blank.

Kinetic Studies:

Supernatant derived from unexposed earthworms was used to evaluate the maximum velocity of the substrate hydrolysis (V_{max}). Michaelis Menten constant (K_m) was estimated by the double-reciprocal method of Linweaver and Burk (1934). Optimum protein concentration and optimum incubation time for the earthworm AChE were also determined.

***In vivo* Effect of Earthworm AChE Activity by Malathion, Dimethoate, Methomyl and Cupper Hydroxide.**

Earthworms were exposed to the concentrations of 0.1, 0.5 and 1 of the LC_{50} of malathion, (7.23, 36.14 and 72.27 mg/kg) dimethoate, (1.08, 5.40 and 10.79 mg/kg) methomyl (0.088, 0.44 and 0.88 mg/kg) and cupper hydroxide (34.64, 173.21 and 346.42 mg/kg). The enzyme sources and the enzyme assay were done as mentioned before. The enzyme activity in the treatments was calculated as a percent of the enzyme activity in the control.

Results were subjected to analysis of variance (ANOVA)(CoStat statistical Software, 1990). The standard error (SE) of three replications was calculated.

RESULTS AND DISCUSSIONS

***In vivo* experiment**

Toxicity of methomyl, dimethoate, malathion and cupper hydroxide on the *L. terrestris* after 7 days of exposure was reported. Median lethal concentrations (LC_{50}s) of the effect of previous pesticides, their confidence limits and slopes were estimated (Table 1). At the LC_{50} levels, methomyl was the most toxic pesticide (LC_{50} = 0.88mg/kg of soil), while cupper hydroxide was the least toxic one (LC_{50} = 346.4mg/kg). In other words the three insecticides were more toxic to *L. terrestris* compared with the tested fungicide (cupper hydroxide).

Assay of AChE

Three different concentrations of each tested pesticides (0.1, 0.5 of LC_{50} and the LC_{50}) were estimated from Table 1. The enzyme protein concentration per

Table1. Toxicity of tested pesticides on the earthworms (*L. terrestris*) after7 days of artificial soil exposure

Pesticides	LC_{50} (mg/kg)	LC_{50} (confidence limits)		Slope	Probability
		Lower	Upper		
Methomyl	0.88	0.73	1.06	1.59	0.06
Dimethoate	10.79	8.82	13.18	2.19	0.56
Malathion	72.27	70.07	80.86	4.41	0.97
Cupper Hydroxide	346.42	332,90	360.50	5.95	0.84

assay was 125ug. The activity of AChE as change in absorption at 412 nm was determined. The substrate concentration was 2 mM. The kinetics of AChE was also determined. The dissociation constant of the enzyme-substrate complex, defined as k_m (Michaels constant) was graphically determined by using Lineweaver Burk Plots of reciprocal substrate concentration (1/S) against reciprocal velocity (1/V). The values of km and V max were 0.0165 mM and 0.01 m mole / min respectively.

Comparative analysis of the k_m appears lower value than that reported by Caselli *et al.*, (2006) (0.14 mM). This explanation of these results might be due to the highly polymorphic AChE enzymes in most species and the number of genes coding for different isoforms varies between species (Bebianno *et al*., 2004).

The effect of the four tested pesticides at the three levels of concentrations on earthworms AChE activity after exposure to contaminated soils for 7 days was illustrated (Fig. 1 and Table 2 A,B). Data showed that inhibition of AChE was increased with increasing the concentrations of pesticides. The maximal inhibition of AChE activity was at the LC_{50} values of insecticides compared with the other two concentrations (0.1 and 0.5 of LC_{50}). Methomyl treatment showed the highest reduction of AChE activity (98.9%) followed by malathion (93.2%) and dimethoate (89.8%) at the LC_{50} levels. In contrast, cupper hydroxide which contains cupper element showed induction of AChE activity at the three levels of LC_{50}, the maximal induction was at 0.5 LC_{50} (27.7) followed with 0.1 (20.3) and the value of LC_{50} (6.8) compared with control. These results may refer that still the concentration of cupper hydroxide at LC_{50} value of cupper hydroxide activate earthworm's AChE, because many elements play as coenzymes in the biological systems.

The data also reflected that AChE activity was strongly decreased by increasing the concentrations of the tested insecticides, which caused significant inhibition. These data confirm that AChE is the target for methomyl, dimethoate and malathion. The inhibition of AChE activity has been considered as sensitive biomarker to assess pesticide effects on various non-target organisms (Damiens *et al*., 2004 and Ferrari *et al*., 2004). The results in line with that reported by Caselli *et al.*, (2006) who found that carbaryl (carbamate insecticide) was able to reduce the earthworm's AChE activity by about 95% when it was used at high concentration (10^{-5} M). Moreover, Rao *et al*., (2003) reported that chlorpyrifos inhibited earthworm's AChE and the inhibition of AChE was a dose and time dependent.

Table (2. A). *In vivo* inhibition of earthworm AChE by methomyl, dimethoate and malathion at different concentrations

Insecticide	Conc.(mg/kg)	S.A ± S.E (ΔO.D/mg protein.min)	Activity (% Control)	% Inhibition
Control	0.0	0.0177 ± 0.0000	100	0.0
Methomyl	0.088	0.0167 ± 0.0003	94.4	5.6 c
	0.44	0.0049 ± 0.0001	28.2	71.8 b
	0.88	0.0002 ± 0.0000	1.1	98.9 a
Dimethoate	1.08	0.0057 ± 0.0003	32.2	67.8 c
	5.40	0.0027 ± 0.0001	15.3	84.7 b
	10.79	0.0018 ± 0.0000	10.2	89.8 a
Malathion	7.23	0.0053 ± 0.0001	29.9	70.1 c
	36.14	0.0037 ± 0.0001	20.9	79.1 b
	72.27	0.0012 ± 0.0000	6.8	93.2 a

Numbers within the same insecticide followed by the same letter are not significantly different.

Table (2. B). *In vivo* induction of earthworm AChE by cupper hydroxide at different concentrations

Conc.(mg/kg)	S.A ± S.E (ΔO.D/mg protein.min)	Activity (% Control)	% Induction
0.0 (control)	0.0177 ± 0.0000	100	0.0
34.64	0.0213 ± 0.0002	120.3	20.3 b
173.21	0.0266 ± 0.0000	127.7	27.7 a
346.42	0.0189 ± 0.0001	106.8	6.8 c

Numbers followed by the same letter are not significantly different.

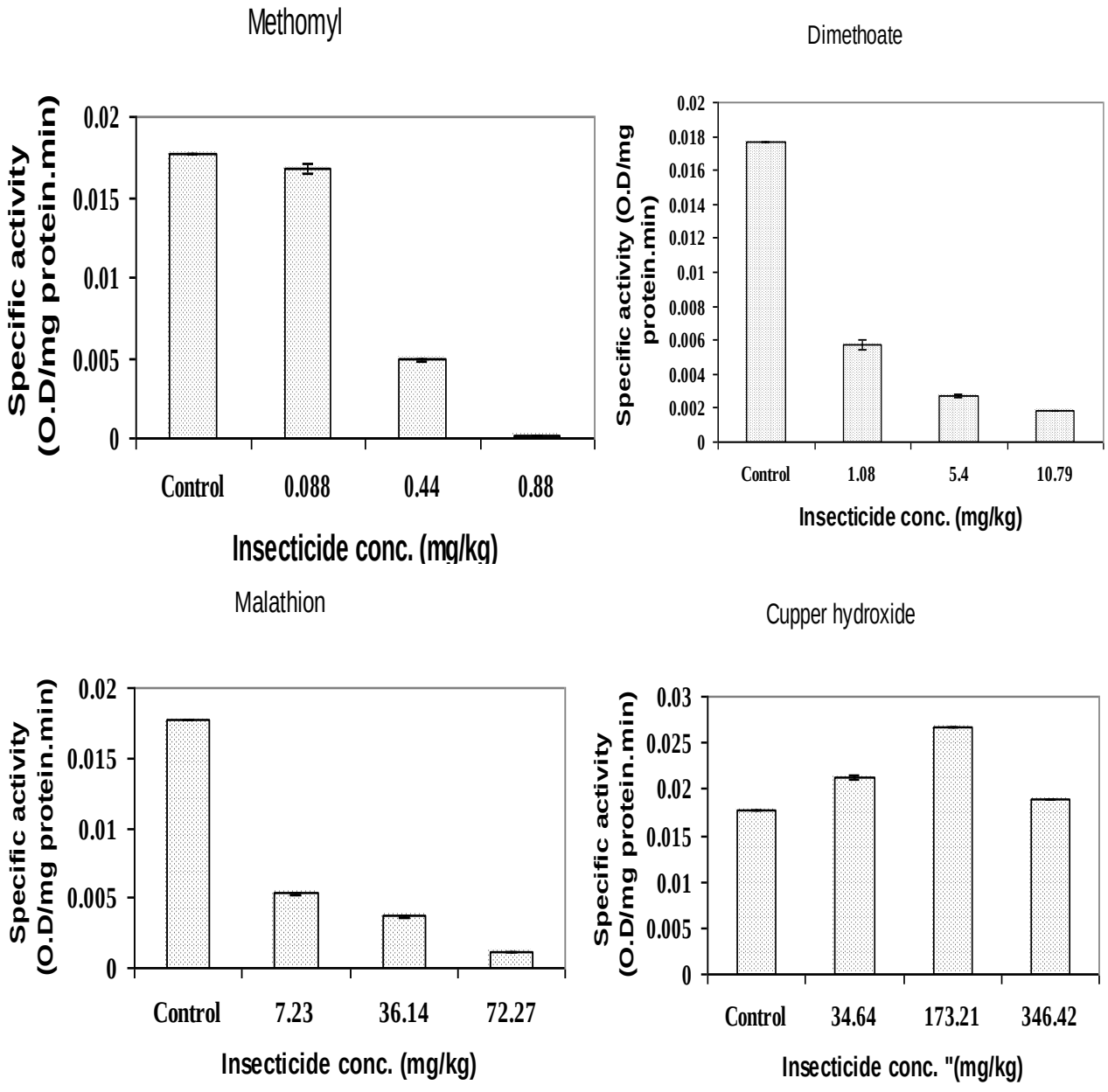

Fig 2. *In vivo* effect of certain pesticides on the AChE activity of earthworm

Biomarker responses have great potential for assessing site pollution because they integrate a wide array of environmental toxicological and ecological factors that control and modulate exposure to contaminants, however many non-pollution related variables may interfere with biomarker response (Arnaud *et al*., 2000). The need to increase our knowledge of biomarker responses in earthworms has been stressed (Kammenga *et al.*, 2000, Sanchez-Hernadez, 2006, Gambi *et al.*, 2007 and Rault *et al*., 2007).

In conclusion, the activity of earthworm's AChE that assessed *in vivo* was dose dependently inhibited by carbamate or organophosphate compounds which might acts as competitive inhibitors. AChE activity isolated from earthworms could be used as a biomarker for insecticide contaminants. Our results further support the use of AChE as a biomarker of pesticide contamination, and could be used for monitoring soil contamination.

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الملخص العربي

الأسيتايل كولين إستيريز لديدان الأرض كمؤشر حيوى لرصد تأثيرات المبيدات

صفاء مصطفى عبد الرحمن

تم تقدير التأثير الداخلى لثلاث مبيدات هى الميثوميل (من مجموعة الكارباميات) والداى ميثويت والملاثيون (من مجموعة الفوسفور العضوية) ومبيد من المبيدات الفطرية المحتوية على عنصر النحاس (كبر هيدروكسيد) على نشاط إنزيم الأسيتايل كولين إستيريز المعزول من دودة الارض لمعرفة أفضل إستجابة لإنزيم الأسيتايل كولين إستريز للكيماويات الحقلية فى ديدان الارض.

وقد أستخدم ثلاث تركيزات من الجرعات القاتلة لـ 50% من الديدان(LC_{50}) للاربعة مركبات والتى تم تقديرها من إختبارات التقييم الحيوى لدراسة تأثيرها على نشاط الإنزيم وهى قيمة LC_{50} ، ونصف وعشر هذه الجرعة.

وقد أدت هذه المبيدات بالثلاث تركيزات المستخدمة و المختبرة الى تثبيط نشاط إنزيم الأسيتايل كولين إستيريز وقد ارتبطت شدة التثبيط مع زيادة التركيز المستعمل فى الثلاث مبيدات الاولى السابقة الذكر. وأظهرت النتائج أيضا أن أعلى تثبيط للإنزيم كان بإستخدام الجرعة القاتلة لـ50% من الديدان وذلك بعد 7 أيام من تعرض دودة الارض للتربة الملوثة بمبيد الميثوميل (98.9%).

أما المبيد الفطرى كبر هيدروكسيد (داكوسيد) والذى يحتوى على عنصر النحاس فقد أظهر تنشيط لإنزيم الأسيتايل كولين إستيريز عند الثلاث مستويات المختبرة من قيم LC_{50} وكان أعلى تنشيط عند إستخدام نصف جرعة LC_{50} متبوعا بقيمة عشر LC_{50} وأخيرا قيمة LC_{50} (27.7%، 20.3%و 6.8)على التوالى وذلك بالمقارنة بالكنترول.

هذه النتائج تعضد إستخدام إنزيم الأسيتايل كولين إستيريز كمؤشر حيوى لرصد تلوث التربة بالمبيدات ضمن المؤشرات الحيوية الاخرى مع إعتبار ديدان الآرض كائن دليلى.