Biological Systems: Open Access

Saadati and Mirzaei, Biol Syst Open Access 2016, 5:2
DOI: 10.4172/2329-6577.1000168

Research Article Open Access

Insecticide-Enzyme Interaction: Cypermethrin, Chlorpyrifos, Diazinon and Deltamethrin with α-Amylase and Lipase in the Gut of Sunn Pest, *Eurygaster integriceps*

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Received date: July 22, 2016; **Accepted date:** October 05, 2016; **Published date:** October 13, 2016

Abstract

Many digestive enzymes were reported from the gut and salivary gland of sunn pest, Eurygaster integriceps. Lipase and amylase are two main enzymes that play key role in the lipid and carbohydrate metabolism. Enzyme activities are affected with biotic and abiotic factors in the *in vivo* and *in vitro* condition. Nowadays, digestive enzymes– insecticides interaction in the insect body is not clear. In this study, the effects of four insecticides, cypermethrin, chlorpyrifos, diazinon and deltamethrin, on the lipase and amylase activity were determined. Our analysis revealed that lipase was more sensitive to insecticides in comparison to the α-amylase. Cypermethrin have showed the maximum inhibitory effects on the amylase and lipase activity in comparison to the others compounds. Chlorpyrifos and diazinon have inhibited digestive enzyme activities in the similar level. Results indicate that lipase and α-amylase are very sensitive to insecticides and were profoundly inhibited by cypermethrin, chlorpyrifos, diazinon and deltamethrin. Accordingly, metabolism of carbohydrates and lipids in the gut of sunn pest will reduce and nutrition process may be suppressed.

Keywords: Enzyme; Gut; Inhibitor; Insecticide; Sunn pest

Introduction

Sunn pest, *Eurygaster integriceps*, is the most important of crop pest in the Iran. It is an oligophagus insect that causes serious damage to wheat and barley. The various methods of pest management such as biological, chemical and ecological controls were used for reducing its losses, but none of them were incredible in the economical agriculture [1,2]. Nowadays, new ways are focusing on the disruption of normal biochemical process like using of enzyme inhibitors and BT crops [3]. These methods considers as safe actions in the pest management, because insecticidal proteins have a minimal risk for the non target organisms.

Extra oral digestion as a powerful method in feeding has evolved in the hemipterous insects [4,5]. In this way, salivary enzymes are injected to the plant tissues and after liquefying, foods pumped to the gut for final digestion [6-10]. Food digestion in the organisms catalyzed with various set of enzymes. Protease, lipase and carbohydrates as critical proteins in the digestive system were reported from gut and salivary glands of the sunn pest [9-12]. Any disruption in the digestive enzymes activity can be lead to stop and/or reduce of the feeding. Different agents like pesticides, plant metabolites, intrinsic regulators, environmental factors (temperature, humidity) can be acted as enzyme disruptors.

Carbohydrates are main food of the phytophagus insects. There are different carbohydrases (α and β-galactosidae, α and β-glucosidae, α-amylase) in the gut and salivary glands of the sunn pest [10,12]. Degradation of starch need to α-amylase (EC 3.2.1.1) activity that breakdowns α-(1, 4) glycosidic bond in the starch and its derivations [13]. This enzyme has addressed for inhibiting with various protein and nonproteins compounds [11]. Inhibiting of gut amylase in the some pest like *Callosobruchus maculates* is reached to practical step in the pest management [3]. Lipids are lowest valuable in comparison to the carbohydrates, but amount of released energy from them is very more than to carbohydrates in the similar volume. Lipase (EC 3.1.1.3) is water-soluble enzyme that causes to hydrolysis of ester bonds in the lipid compounds into free fatty acids and glycerol [14]. Lipase and α-amylase have many isozymes in the insect world particularly in the midgut of sunn pest [10]. Hence they can be used as potential candidates in the field of enzyme inhibiting.

Chemical control is commonplace application in the sunn pest management in Iran [2]. Different insecticides which belonged to various chemical groups are recommended to *E. integriceps* management. Organophosphate and pyrethroids compounds are main selection of farmers because they have known as speed and efficient chemicals. On the other hand, environmental pollution especially adverse effects on the non target organisms created serious concerns for now and future. New researches are needed to investigate about effective ways to reduce ecological risks of the chemicals. Insect-insecticide interaction, as dynamic system, increases our knowledge to improve chemical application. Although modes of action in the most insecticides are obvious (direct action) but the other targets which may be affected after treatment are not determined (indirect action). To elucidate the effects of insecticides on the nutrition process, digestive enzyme- insecticide interaction experiments should be used as *in vivo* and *in vitro* system.

In this study, for the first time, interaction of lipase and α-amylase from the gut of adult sunn pest with cypermethrin, chlorpyrifos, diazinon and deltamethrin were considered as *in vitro* experiments. We undertook this study to gain a better understanding of digestive enzyme behaviors after exposure to different concentrations of

chemicals. Also, we hope that result of these research lead to new opportunities for finding of new enzyme inhibitors and increase our knowledge about side effects of some insecticides on the digestive enzymes activities.

Material and Methods

Insects

Overwintering adults, *E. integriceps* (Hemiptera, Scutelleridae) were collected from Birjand, Iran, in February 2015 and reared in an insectary room on wheat, *Triticium aestivum* L. Poales: Poaceae) variety Bam at 27°C with a 16:8 L: D photoperiod.

Sample preparations

The midgut of adult insects were dissected under a stereomicroscope in ice cold phosphate buffer (4°C, pH=6.9). The midguts were separated from the insect bodies, rinsed in ice-cold phosphate buffer and three number placed in a microtube containing one ml of cold phosphate buffer. The midguts were homogenized by using a homogenizer immediately after dissection. The homogenates were centrifuged at 12000 rpm for 10 min at 4°C. The supernatants were stored at -20°C for later analyses.

Four insecticides contain cypermethrin (40 EC, Partonar Co., Iran), chlorpyriphos (40.8 EC, Aria Shimi Co., Iran), diazinon (Ghazal Shimi Co., Iran) and deltamethrin (2.5 EC Partonar Co., Iran) were ordered for this experiments. After preliminary test 2500, 2000, 1500, 1000, 500, 100 and 0 ppm (active ingredient) were selected for interaction with enzymes. 50 µl enzyme solution with 450 µl toxic solutions incubated 30 min in the room temperature before enzyme assay.

Enzyme assay

α-Amylase activity assay

Amylase activity in the midgut was determined using a diagnostic kit (Amylase kit®, Pars Azmoon Co., Iran). The substrate was ethylidene-pnitrophenyl maltoheptaoside (EPS-G7). Absorbance, which is directly related to α-amylase activity, was measured at 405 nm and 37°C using an auto analyzer (Alcyon 300® Plus, Molecular Devices Corporation, Sunnyvale, CA). Before application, the auto analyzer calibrated with the control sera N and P (TrueLab N® and TrueLab P®, respectively; Pars Azmoon Co., Iran) and a calibrator solution (TrueCal U®, Pars Azmoon Co., Iran). After calibration, the auto analyzer mixed 4 µl of enzyme sample with 300µl of substrate solution, automatically, and calculates the enzyme activity (IU/L) after a reaction delay of 1 min and 36 s. Optimized pH was eight for all treatments. The assays were replicated three times. Finally, the specific α-amylase activity calculated as U/mg protein that known Specific activity.

Lipase activity assay

Lipase activity in the midgut was determined using a diagnostic kit (Lipase kit®, Biorexfars Co., Iran). The substrate was 1, 2-o-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) (DGGR). Absorbance, which is directly related to production of methylresorufin and lipase activity, was measured at 580 nm and 37°C using an auto analyzer. Optimized pH was eight for all treatments. Recommended calibrators and the other considerations were similar to amylase assay procedure.

Total protein assay

Total protein were assayed according to Biuret test using diagnostic kit (Total protein kit®, Pars Azmoon Co., Iran). In this procedure, copper sulfate ions react with the peptide bonds to produce purple (or pink) color. The final amount of proteins in the samples is estimated by comparing their intensity color with standard solution that contains defined bovine serum albumin (BSA) concentration.

Statistical analysis

Data were compared by one-way analysis of variance (ANOVA) and factorial design that followed by Duncan's studentized test at $p<0.05$ (Mstat-C and Spss ver. 15). Differentially activities were shown (as different letters) in figures.

Results

α-amylase- insecticide interaction

Mixture of enzyme solution and different concentrations of insecticides were incubated for 30 min in temperature room before determination of enzyme activity. Results showed that there was a significant difference among different insecticides and their concentrations (Figure 1). The highest level of enzyme activity was occurred in the 1000 ppm of chlorpyrifos and deltamethrin and 0 ppm (control). On the other hand, 2500, 2000 ppm of deltamethrin and 2500 ppm cypermethrin were caused the minimum of enzyme activity (Figure 1). Trend of enzyme inhibiting for cypermethrin was regular as the highest and lowest inhibiting were observed in the maximum (7.07%) and minimum concentrations (92.83%), respectively (Table 1). This trend also was observed in the diazinon treatment with 39.89% and 96.24% (Table 1).

	Cypermethrin	Chlorpyrifos	Diazinon	Deltamethrin
Concentration ppm	Enzyme activity (% control)			
100	92.83	95.09	96.24	77.2
500	70.7	97.87	72.33	37.96
1000	41.1	109.49	83.15	155.33
1500	27.72	87.59	80.51	63.38
2000	16.81	78.15	64.24	0

2500	7.07	32.23	39.89	0

Table 1: Effects of six concentrations of four insecticides on the α-amylase activity in the midgut of sunn pest 30 min after incubation. Values are average of Inhibitory effects and calculated based to the control treatment (% inhibitory).

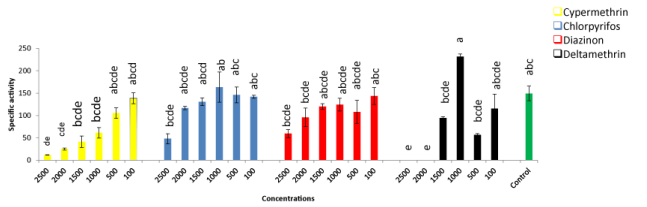

Figure 1: α-amylase activity after incubation with different concentration of insecticides in the Adult sunn pest. Values are the average specific activity ± SE from three independent experiments. Different letters showed significantly difference among concentrations (P<0.05).

Significant difference between pyrethriod and organophosphate chemicals was observed as the rate of enzyme activity after chlorpyrifos and diazinon treatments were more than that of cypermethrin and deltamethrin treatments (Figure 2). Also, there was significant difference between cypermethrin and deltamethrin as the amount of enzyme activity in deltamethrin was more than that in cypermethrin (Figure 2). There was no significant difference between clorpyriofos and diazinon. In comparison among different concentrations 1000, 100 and 0 ppm; 1500, 500; 2000 and 2500 were caused the highest of amount enzyme activity, respectively (Figure 3). Although all used concentrations of insecticides inhibited amylase activity but chlorpyrifos and deltamethrin in 1000 ppm were acted as activator for this enzyme.

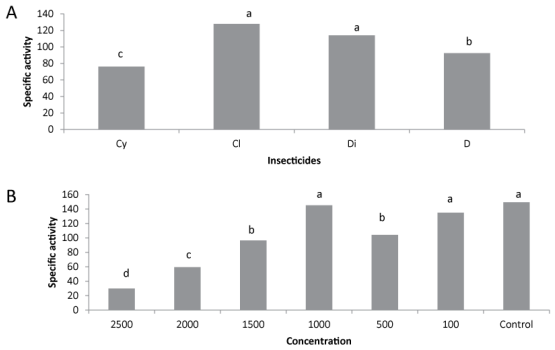

Figure 2: Enzyme activity after incubation with four insecticide (A) and Different concentration in the Ault sunn pest (B). Values are the average specific activity ± SE from three independent experiments. Different letters showed significantly difference among insecticides and their concentrations (P<0.05).

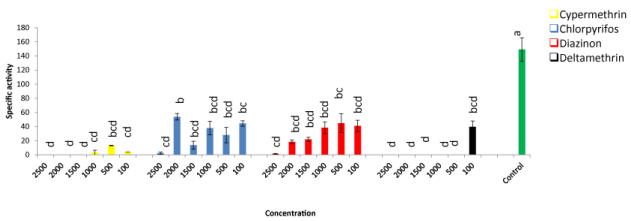

Figure 3: Lipase activity after incubation with different concentration of insecticides in the Adult sunn pest. Values are the average specific activity ± SE from three independent experiments. Different letters showed significantly difference among concentration (P<0.05).

Lipase- insecticide interaction

Mixture of enzyme solution and different concentrations of four insecticides were incubated for 30 min in temperature room before measuring of enzyme inhibitory. There were significant differences among various insecticides and their concentration (Figure 4). The highest level of enzyme activity was observed in the 0 ppm (Control treatment) (Figure 4). On the other hand, 2500, 2000 and 1500 ppm from cypermethrin and deltamethrin; 1000 and 500 ppm deltamethrin were caused the level lowest of enzyme activity (Figure 4). Trend of enzyme inhibiting for deltamethrin was regular as the highest and lowest inhibiting values were observed in the maximum (0%) and minimum concentration (26.66%), respectively (Table 2). This trend was not observed for the other insecticides (Table 2). There was a significant difference between pyrtheriod and organophosphate chemicals as the rate of enzyme activity after chlorpyrifos and diazinon treatment were more than that of cypermethrin and deltamethrin (Figure 4A). But, there was no significant difference between cypermethrin with deltamethrin and chlorpyriofos with diazinon (Figure 4A). As a whole, in comparison among different concentrations, 0 and 2500 ppm were caused the highest and lowest of enzyme activity, respectively (Figure 4B).

Concentration ppm	Cypermetrin	Chlorpyrifos	Diazinon	Deltamethrin
	Enzyme activity (% control)			
100	2.76	29.75	27.46	26.66
500	8.91	18.83	30.05	0
1000	2.2	25.44	25.69	0
1500	0	9.16	14.67	0
2000	0	36.09	12.35	0

2500	0	1.62	1.02	0

Table 2: Effects of six concentrations of four insecticides on the Lipase activity in the midgut of sunn pest 30 min after incubation. Values are average of Inhibitory effects and calculated based to the control treatment (% inhibitory).

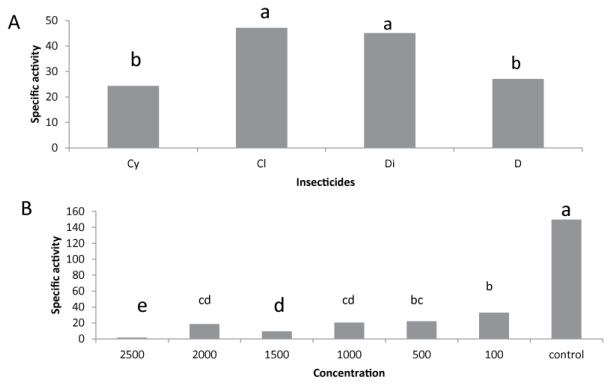

Figure 4: Enzyme activity after incubation with four insecticide (A) and Different concentration in the Ault sunn pest (B). Values are the average specific activity ± SE from three independent experiments. Different letters showed significantly difference among insecticides and their concentration (P<0.05).

Discussion

Tremendous benefits have reported from the use of insecticides in agriculture, medicine and veterinary. Our knowledge about direct and indirect effects of insecticides is not complete and need to increase continuously. Although it is proved that main targets of organophosphate and pyrethroid pesticides are acetylcholinesterase and sodium channels, but it seem that set of various proteins in the target organism affected by pesticides. Saadati et al., [10] showed that proteome analysis of digestive system in the sunn pest can be used as appropriate technique for studying of changed proteins after exposure to the specific treatment. Sharma et al., [15] showed that some of insecticides reduced expression of effective proteins in the carbohydrates metabolism in the brown plant hoppers. Widespread use of insecticides for sunn pest management crated concern about environmental pollution, insect resistance and unwanted risks. Hence, focusing on the new aspects of insecticides effects on the target and non target organisms may be led to safer using them in the environment. Occurrence of the side effects of insecticides on the nutrition process of insects can be used as secondary factor to help us for choosing of suitable insecticide and their doses. Digestive enzymes as core of biochemical process are potential targets because any disrupt to their activity may be led to reducing or suppressing of nutrition [16]. Two important enzymes, α-amylase and lipase were reported from gut of sunn pest [9,10]. These enzymes play key roles in the carbohydrates and lipid metabolism. In the present study, these enzymes were incubating with different doses of known insecticides, which recommended for sunn pest control in Iran, to study amount of changes on their activities.

As a whole, results showed that amylase and lipase activity in the gut of sunn pest were inhibited by cypermethrin, chlorpyrifos, diazinon and deltamethrin. In this study, it was further found that the amylase activity was inhibited strongly at higher concentrations in comparison with lower concentrations. Amylase activity was decreased in the all treatment exceptionally 1000 ppm concentration of chlorpyrifos and deltamethrin in comparisons to the controls. It is not possible to describe a reason/s for this behavior but it is guessable that enzyme conformation was changed in this condition because all active sites of the enzyme were not blocked with this inhibitors.

Maximum inhibitory of digestive amylase were occurred in the high concentrations of cypermethrin and deltamethrin more than that in chlorpyrifos and diazinon. This result suggests that pyrethroid insecticides have more inhibitory effects on the digestive enzyme in comparison to the organophosphate compounds. The inhibition of amylase and lipase by the pyrethroid compounds could be mainly due to the presence of Cyanide factor. Amylase activity completely removed in the 2000 and 2500 ppm of deltamethrin. Removing of α-amylase activity in the gut of sunn pest led to accumulation starch and similar carbohydrates and this process may be followed insect dying because of absence energy production mechanism. Cypermethrin and deltamethrin have high octanol-water coefficients and this may be effective in bonding of these compounds to different enzymes like amylase. Reducing of amylase activity after interfering with insecticides led to decrease of insect ability in the starch digestion. A number of different proteinous and nonproteinous inhibitors were reported against α-amylas in the salivary glands of sunn pest and the other insects [11]. Eraslan et al. [17] reported that inserting of deltamethrin to food of mice was not reduced amylase activity in the serum. Also their results showed that deltamethrin reduced acetylcholinesterase in the serum of mics. Amylase activity in the serum of rat showed no appreciable changes after treatment with cypermethrin [18]. Also, amylase activity in the serum of *Gallus domesticus* had not showed significantly change after cypermethrin treatment [19]. Occurrence of different sensitivity of amylase to the insecticides was related to various factors like intrinsic difference of protein sequencing and structure, nature of insecticides, different doses, age and health of organism and environmental factors (Temperature, humidity, pH).

Lipase was classified as serine hydrolyses those breakdown lipids as energy sources. These enzymes have key role in the physiological process like lipid metabolism and transport, regulation of plasma membrane lipid and cell signal transduction [20]. Result of this study indicates that lipase was more sensitive in comparison to the amylase against applied insecticides. For example, deltamethrin completely inhibited lipase activity in all concentration except 100 ppm. In the 1500, 2000 and 2500 ppm of cypermethrin was not recorded any lipase activity, too. But in the all concentration of chlorpyrifos and diazinon lipase activity was recorded. Deltamethrin was caused that amount of triglycerides was increased in the blood serum of the mice [17]. It is possible that main factor for its was inhibitory effects of deltamethrin on the lipase.

Gunes and Yerli [21] reported that deltamethrin inhibited lipase activity in the *Poecilia reticulate*. Pyrethroids type II like cypermethrin and deltamethrin have Cyanide factor in their structures. Cyanides are strong inhibitors for different enzymes in the live cells. They can bind to enzymes and after changing enzyme conformations, convert them to inactive form [18]. Hence, it is rational that pyrethroid compounds have more inhibitory effects in comparison to the organophosphate

compounds. Organophosphate compounds such pesticides were reported as potential lipase inhibitors in the vertebrate animals [22]. Our results proved that chlorpyrifos and diazinon are strong inhibitors for insect lipase, too.

Although, complete inhibition of target enzymes were considered as successful results but even partial inhibition recommended as substantial strategy for insect control. Data from this study indicated that insecticides can inhibit digestive enzyme activity, generally. Also, pyrethroid chemicals were introduced as stronger inhibitors for digestive enzymes in comparison to organophosphate chemicals. Also, enzymatic assays with energy-metabolism enzymes, including Lipase and amylase, after mixture with insecticide showed that effects of insecticides is not limit to the one target and set of different proteins may be affected after treatment.

One of the major challenges is to link the results from *in vitro* studies to *in vivo* condition. The *in vivo* studies are very complicate and need to consider various factors to gain desirable results. We will focus on the enzyme-insecticide interaction to elucidate correlation between *in vivo* and *in vitro* condition for insect systems. Although, there are a few studies that investigate the effects of insecticides on digestive enzymes of sunn pest, new researches should be focus to clear mechanism of inhibitory effects and their interaction with assimilation process.

Acknowledgement

We thank Mrs. Yosefi for technical help and Auoanalyzer setup. We also appreciate M. Farkhari for helping in insect collection and insecticides handling. The authors thank the University of Birjand and Entomological Society of Iran.

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