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Research Article

Investigation of the Effects of Diazinon and Carbaryl Pesticides on Lipase Under *In vitro* Conditions

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ABSTRACT

It is known that the enzyme activities of non-target organisms are influenced *in vivo* or *in vitro* by pesticides that play a major role to obtain higher yields in the field of agriculture. It is foreseen that the pesticides used for various purposes may negatively affect lipase, an enzyme of the digestive system, of the non target organisms. Therefore, in this study, *in vitro* effect of diazinon (organophosphate class) and carbaryl (carbamate class) pesticides, which are known to be widely used in agriculture, was investigated and an effective dosage range of these pesticides on lipase enzyme was determined. It was observed that the lipase enzyme treated with diazinon and carbaryl in the concentration range of 100-2500 ppm was inhibited. Lipase enzyme activity at maximum diazinon concentration was calculated as 2.05%. Carbaryl completely inhibited lipase at maximum concentration The Kinetics conformed to the Michaelis-Menten model and a Lineweaver-Burk graph of the lipase was drawn. Carbaryl showed the strongest inhibitory effect against lipase (K_i: 460.96±28.25 mM; IC_{50}: 2.5±1.3 µM) in comparison to diazinon. In the case of diazinon, K_i and IC_{50} value were found as 481.32±45.18mM and 3.6±0.9 µM for the lipase, respectively. The results showed that *C. rugosa* lipase are inhibited by diazinon and carbryl in vitro. It was shown that the catalytic activity of *C. rugosa* lipase is inhibited competitively by carbaryl but noncompetitively by diazinon

Keywords: *Lipase, Diazinon, Carbaryl, Pesticide, Inhibition kinetics*

Diazinon ve Karbaril Pestisitlerinin Lipaz enzimi Üzerine Etkisinin *In vitro* Koşullarda İncelenmesi

ÖZET

Tarım alanında daha yüksek verimde ürün elde etmek için kullanılan pestisitlerin *in vivo* veya *in vitro* koşullarda hedef olmayan organizmaların enzim aktivitelerini etkilediği bilinmektedirler. Hedef olmayan organizmaya etki eden pestisitlerin sindirim sistemi enzimi olan lipaz enzimini olumsuz şekilde etkileyebileceği öngörülmüştür. Bu nedenle bu çalışmada tarım alanında yaygın kullanıldıkları bilinen diazinon (organofosfat sınıfı) ve karbaril (karbamat sınıfı) pestisitlerinin lipaz enzimi aktivitesi üzerine *in vitro* etkisi araştırıldı ve bu pestisitlerin lipaz enzimi üzerine etkili dozaj aralığı tespit edildi. 100-2500 ppm konsantrasyon aralığında diazinon ve karbaril ile muamele edien lipaz enziminin inhibe olduğu gözlemlendi. Maksimum Diazinon konsantrasyonunda lipaz enzim aktivitesi %2,05 olarak hesaplandı. Karbaril ise lipazı maksimum konsantrasyonda tamamen inhibe etti. Diazinon ve karbaril ile muamele edilen lipaz enzimi için Michaelis-Menten ve Lineweaver-Burk grafiği çizildi. Karbarilin, diazinon ile karşılaştırıldığında lipaz enzimi (K_i: 460.96 ± 28.25 mM; IC_{50}: 2.5 ± 1.3 uM) için daha güçlü inhibitör etki gösterdiği belirlendi. Diazinon ile muamele edilen lipaz enziminin K_i ve IC_{50} değeri sırasıyla 481,32 ± 45,18mM ve 3,6 ± 0,9 uM olarak bulundu. Sonuçlar, *C. rugosa* lipazının *in vitro* olarak diazinon ve carbryl tarafından inhibe edildiğini gösterdi. *C. rugosa* lipazının katalitik aktivitesinin karbaril tarafından yarışmalı bir şekilde, ancak diazinon tarafından yarışmasız bir şekilde inhibe edildiği gösterildi.

Anahtar Kelimeler: *Lipaz, Diazinon, Karbaril, Pestisit, İnhibisyon Kinetiği*

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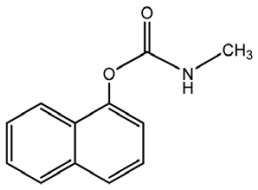
I. INTRODUCTION

Pesticides (especially organophosphorus and carbamates) are the most toxic compounds and are widely used as pest controllers in agriculture [1], [2]. Pesticides can be classified in many different ways according to physical structures, formulations, chemical composition of pesticides, toxicity, usage technique, pesticide function and pest organism they kill. In the classification of pesticides, the classification according to chemical structure and pest organism they kill is more commonly used. Based on chemical structure, pesticides are classified into four main groups namely; organochlorines, organophosphorus, carbamates and pyrethroids [3]. According to pest organism they kill, pesticides are classified as fungicides, insecticides, rodenticides, herbicides, garden chemicals and household disinfectants [4]. Carbaryl and diazinon used in this study are only a few of these pesticides. Carbaryl (1-naphthyl methylcarbamate) is an insecticide in the carbamate family [5] and diazinon is an organophosphate insecticide [6]. Carbaryl is used around the world to control insects on forests, nuts, fruit, citrus, cotton and etc. Also, diazinon is used to control insects on fruit, vegetable field and plants. Organophosphate insecticides have the same toxicity mechanism as the carbamate insecticides [7]. It is known that pesticides can be acted as enzyme disruptors [8]. In particular, they are known to be acetylcholinesterase (AChE) inhibitors [9],[10]. Pesticides can reversibly or irreversibly bind covalently with the serine residue in the active site of AChE [7]. Also, lipase enzyme has serin residue in its active site, so that pesticides can bind and inhibit to lipase. Due to the properties of pesticides to inhibit enzymatic activity, they play an important role in the inhibition studies. To elucidate the effect of pesticides on enzyme activity, *in vivo* and *in vitro* studies should be performed. In this study, for the first time, the inhibition type of carbaryl and diazinon pesticides on *C. rugosa* lipase, one of the digestive system enzymes, was studied as *in vitro*. We undertook this study to understand of *C. rugosa* lipase enzyme behaviors after exposure to different concentrations of carbaryl and diazinon. 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 pesticides on the enzymes activities.

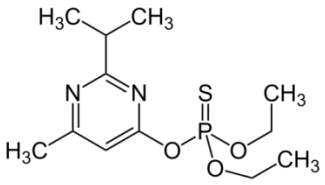
II. MATERIALS and METHODS

A. CHEMICALS

Lipase from *C. rugosa* (CAS Number 9001-62-1) was supplied by Merck KGaA. Pesticides, Carbaryl (CAS Number 63-25-2) and Diazinon (CAS Number 333-41-5), were supplied by Merck KGaA. Also, the other chemicals were purchased by Merck KGaA and prepared analytically.



Carbaryl



Diazinon

Figure 1. *The chemical structures of pesticides: Carbaryl and diazinon*

B. METHODS

B. 1. Lipase Activity Assay

Lipase was dissolved in cold $CaCl_2$ solutions (5 mM) at the concentration of 2.0 mg/mL, immediately before use. To prepare substrate solution, triolein dispersed in 3% (v/v) gum Arabic containing 2.5 mM desoxycholate (DOC) solution was mixed by swirling and adjust to pH 8.0 at 37 °C with 10 mM

NaOH. Enzyme solution (10 µL) was added to 15 mL of the substrate solution. The final concentrations of triolein in the reaction mixture ranged from 0.5 to 4.0 mM. The reaction was run 1-5 min. Small volumes (0.025 mL) of 10 mM NaOH was added to reaction medium. The volume of NaOH used to maintain the pH at 7.7 and the time required were recorded [11].

One unit lipase enzyme defined as the amount of enzyme hydrolyze 1 µmole of triolein per minute at pH 7.7 at 37°C.

B. 2. Effects of Pesticides on Lipase Activity

Diazinon and carbaryl stock solutions (in ethyl alcohol) were prepared. Diazinon and carbaryl were dissolved separately at six concentrations in ethyl alcohol. Final diazinon and carbayl concentrations were 100-2500 ppm in reaction medium. Lipase activity was measured without the pesticides and accepted as 100%. For the inhibition studies, 1 mL of each pesticide was mixed with lipase enzyme solutions. The mixture was incubated for 10 min and then the lipase activities were measured. For determination the effect of ethyl alcohol, 1 ml of ethyl alcohol was added to lipase enzyme solution and lipase activity was measured and the value was subtracted from the lipase activity value obtained after pesticide inhibition. The Kinetics conformed to the Michaelis-Menten model and Lineweaver-Burk graphs of lipase were drawn by using the obtained results (Figure 2 and Figure 3). To identify the inhibition type, K_i, K_i' (dissociation constant of free enzymes and enzyme-substrate complex, respectively) and IC_{50} (concentration of compound/sample required to inhibit 50 % enzyme activity) values were calculated [12]. K_i and K_i' were calculated from the following equations [13].

$$\frac{K'_m}{V'_{max}} = K_m.\frac{1+[I]}{V_{max}} \quad (1)$$

$$V'_{max} = \frac{V_{max}}{(1+\frac{[I]}{K_i})} \quad (2)$$

In the presence of the pesticides, Km' and Vmax' are the values. [I] is the inhibitor concentrations. These calculations were done for each inhibitor concentration (2).

B. 3. Total Protein Assay

The protein concentration was determined using the method of Bradford [14]. For the preparation of Commassie Brilant Blue G-250 solution 100 mg Commassie Brilant Blue G-250 was dissolved in 50 mL 95% ethanol. 100 mL of 85% phosphoric acid was added on it. It was filtered with filter paper and completed to 1L with pure water. For preparation of stock and standard protein solutions 100 µg BSA was completed to 1000 µL with 0.15 M NaCl solution. Standard solutions were prepared 10-90 µg/ 1,5 mL from this stock solution (diluted by 0.1M NaCl solution). 1.5 mL of the prepared standard solutions was taken and 1.5 mL of Commassie Brillant Blue G-250 solution was added to them, and the absorbance values at 595 nm against blank were read in 3 mL cells. 1.5 mL 0.15 M NaCl and 1.5 mL Commassie Brilant Blue G-250 were used as a blank. Each experiment was repeated 3 times and averaged. A working graph was drawn with the absorbance values obtained.

III. RESULTS and DISCUSSIONS

A. EFFECTS of PESTICIDES on the LIPASE ACTIVITY

Lipase activity was measured in the presence of carbaryl and diazinon (100-2500 ppm). Results showed that the inhibition of enzyme increased with increasing concentrations of the pesticides from 100 to 2500 ppm. The highest level of enzyme activity was observed in the 0 ppm (none pesticide, control) as shown in Table 1. In the minimum concentration of carbaryl (100 ppm), lipase showed 24.18±0.18 % activity. For 500 ppm carbaryl, 12.17±0.51 % lipase activity was obtained. On the other hand, 1000-2500 ppm of carbaryl inhibited the lipase completely. Trend of enzyme inhibiting for diazinon was regular as the highest and lowest inhibiting were observed at 500 ppm (32.45±0.65%) and 2500ppm (2.05±0.15%). Activities of lipase treated with pesticides (%) were shown in Table 1. When the literature studies are examined, it was seen that the effects of carbaryl and diazinon on lipase enzyme were studied before by G. M. Christensen and B. Riedel [15] but at the first time inhibiton kinetic and inhibition type of these pesticides on *C. rugose* lipase was studied in our study. However, Mohammad Saadati and Mostafa Mirzaei observed 96.24-39.89 % lipase activity in the Gut of Sunn Pest, *Eurygaster integriceps* for diazinon (100-2500 ppm), respectively [16]. In agreement with these results, it can be said that diazinon effectively inhibits lipase enzyme.

Table 1. *Effects of six concentration of diazinon and carbaryl insecticides on C. rugosa lipase activity after 10 min incubation. Values are average of inhibitory effects and calculated based to the control treatment (%).*

Concentration (ppm)	***Diazinon***	***Carbaryl***
	Enzyme activity (%)	
[a]None	*100±2.12*	
100	*28.32±0.21*	*24.18±0.18*
500	*32.45±0.65*	*12.17±0.51*
1000	*23.25±0.82*	*0*
1500	*16.67±0.11*	*0*
2000	*11.22±0.28*	*0*
2500	*2.05±0.15*	*0*

The data are presented as mean value±standard deviation of triplicate analyses. [a]Reference (none pesticide) condition set as 100% activity

B. KINETIC PARAMETERS

To compare the inhibitory capacity of carbaryl and diazinon, IC_{50} values were determined (Table 2). Diazinon had the highest IC_{50} (3.6±0.9 µM) value. IC_{50} value of carbaryl was determined as 2.5±1.3 µM. Only, Dinh Thien Phuong [17] observed that carbaryl had highest IC_{50} (30 µM <) for lipase *in vivo*.

The effects of carbaryl and diazinon on the enzyme kinetics of *C. rugosa* lipase using triolein as substrate were evaluated, and results are shown in Fig. 2 and Fig. 3, respectively. To characterize the inhibition type of each pesticide, according to lineweaver-burk graphs of lipase treated with carbaryl and diazinon (Fig.2 and Fig.3), V_{max} and K_m were calculated at different concentrations of the pesticides and are shown in Table 2. So that, both linear and non-linear analyses of inhibition curves of each pesticide were performed. As shown in Fig.2, carbaryl has high K_m and low V_{max} values compared to the control; linearized plots indicated a mixed-type inhibition. On the other hand, in Fig.3, diazinon possessed significantly higher apparent K_m and V_{max} values than control; linearized plots indicated a mixed-type inhibition.

Ki', Ki and Vmax values were calculated and shown in Table 2. Ki values of the carbaryl and diazinon were observed higher than Ki'. These higher Ki values indicate that the inhibitor has more affinity for the enzyme-substrate complex than for the free enzyme in a mixed inhibition mechanism [18].

Table 2. *Catalytic parameters (V_{max}, K_m, K_i' and K_i) and IC_{50} of C. rugosa lipase in the presence of different carbaryl and diazinon*

Compounds	*C (mM)*	*V_{max} (µmole/dk)*	*K_m (mM)*	*K_i (mM)*	*IC_{50} (µM)*
[a]None	*0.5 - 4.0*	*27.02±2.21*	*1.88±0.12*	*n.d*	*n.d*
Carbaryl	*2.48*	*17.24±1.58*	*15.77±2.42*	*460.96±28.25*	*2.5±1.3*
Diazinon	*3.28*	*2000±59.42*	*178.5±13.56*	*481.32±45.18*	*3.6±0.9*

The data are presented as mean value±standard deviation of triplicate analyses. n.d.=not determined. [a]Reference (none pesticide) condition set as 100% activity.

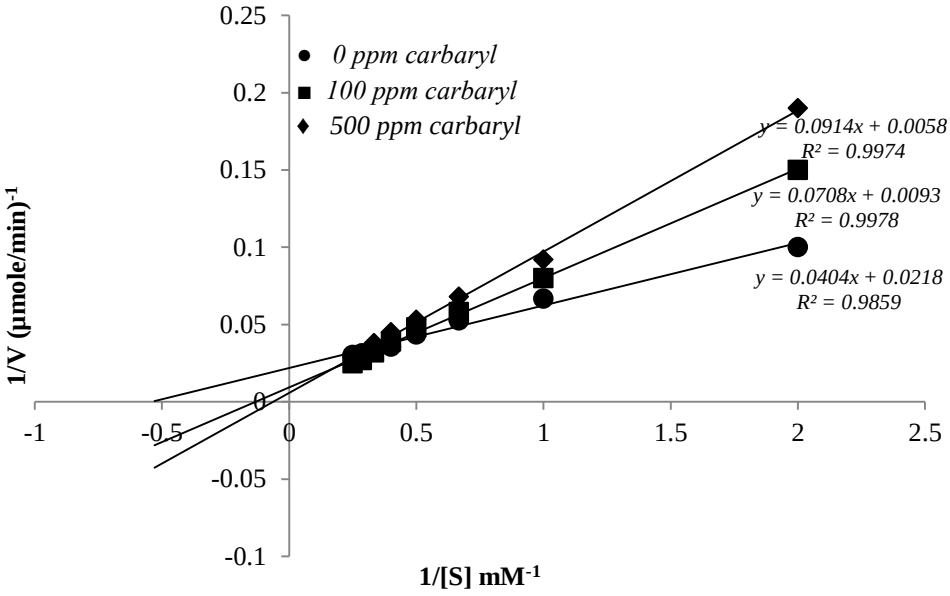


Figure 2. *Lineweaver-Burk graph of lipase treated with carbaryl.*

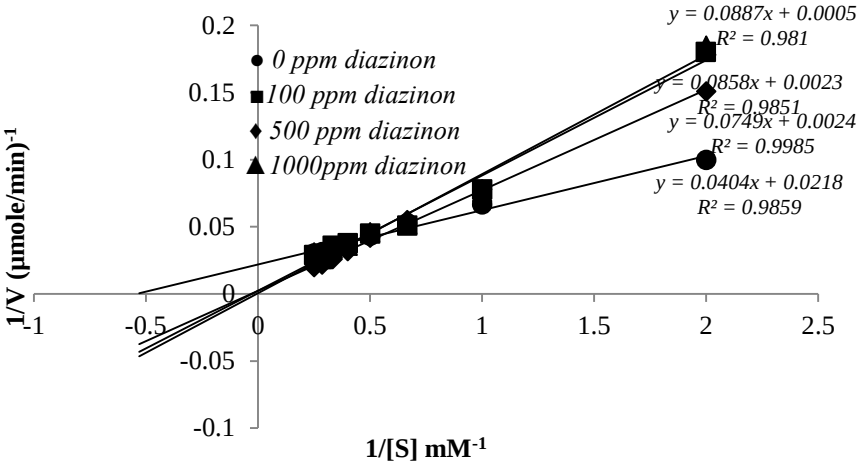


Figure 3. *Lineweaver-Burk graph of lipase treated with diazinon*

IV. CONCLUSION

To investigate the effects of diazinon and carbaryl pesticides on the *C. rugosa* lipase, lipase activity (none pesticide) and pesticide treated lipase activity was measured by using titrimetric method. Based on the experimental results, the inhibition of enzyme increased with increasing concentrations of the pesticides from 100 to 2500 ppm. The highest inhibition was observed with carbaryl from 1000 ppm to 2500 ppm (100% inhibition) and according to obtained K_i and IC_{50} values for carbaryl treated lipase, carbaryl showed the strongest inhibitory effect against *C. rugosa* lipase (K_i: 460.96±28.25 mM; IC_{50}: 2.5±1.3 µM) in comparison to diazinon (K_i: 481.32±45.18mM; IC_{50}: 3.6±0.9 µM). The results showed that *C. rugosa* lipase are inhibited by diazinon and carbryl *in vitro*. It was shown that the catalytic activity of *C. rugosa* lipase is inhibited competitively by carbaryl but noncompetitively by diazinon. Since this study is the first study on inhibition kinetics and inhibition type of *C. rugosa* lipase by carbaryl and diazinon pesticides, we hope that this study is a reference for other related studies to be done in the future.

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