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Effect of Some Pesticides on Chitinase Enzyme Extracted From Different Insects .

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ABSTRACT

Chitinase (poly -1,4- (2-acetamido -2- deoxy) – D - glucoside Glycanohydrolases EC 3.2.1.14) was extracted from Culex pipenes, Spodoptera littoralis larval stage and Musca domestica, Periplaneta americana adult stage. The optimum condition for maximum chitinase activity were, at 4.0 pH , 35 ° C as a reaction temperature using chitin (an insduble linear B-1,4-linked polymer of N -acetylglucosamine) as enzyme substrate .

An attempt was made to evaluate the ability of the tested Pesticides to inhibit the chitinase activity from different sources. The I_{50} values of diuron were $4x10^{-5}$, $3x10^{-5}$, $3.7x10^{-5}$ and $6x10^{-5}$M for S. littoral, P. americana, M. domestica and C. pipienes, respectively, and the I_{50} values of chloropropham were $3.7x10^{-5}$, $4.6x10^{-5}$, $5.1x10^{-5}$ and $1x10^{-4}$M for S. littoralis, P. americana, M. domestica and C. pipienes respectively. However propanil incrase the chitinase activity more than control. On the other hand the percent of chitinase inhibition by deltametrin, methocarb, aldicarb, paraoxon and DNOC were 21,20,18,17 and 15 respectively.

The data shows interactive of different compounds studied on some biological parameters of treated insects depending mainly on the effect of these compounds on the chitin biosynthesis.

INTRODUCTION

Chitin an insoluble linear B-1,4-linked polymer of N-acetylglucosamine,is a common constituent of fungal cell walls and of the exoskeletons of arthropods.All organisms that contain chitin also contain chitinase (poly-B-1,4-(2-acetamido-2-deoxy)-D-glucoside glycanohydrolases, EC 3.2.1.14),which are presumably required for morphogenesis of the cell walls and exoskeletons (Gooday,1977),.Other organisms that do not contain chitin may produce chitinase to degrade the polymer for food, e.g. soil bacteria that secrete chitinase in response to chitin in their environment (Oranusi and Trinci,1985)and the digestive tract of fish (Danulat and kaursch,1984).Plants also have been found to contain chitinase, often after enzyme synthesis has been induced by microbial infections or other injuries (Pegg and Young,1982; Boller et al , 1983; Boller, 1985) . Since plants do not contain chitin in their cell walls, it has been postulated that they produce chitinase to protect themselves from chitin-containing parasites (fungi,insects) (Abeles et al ., 1970 ; Bell, 1981; but direct evidence supporting this hypothesis has been laking (Boller, 1985) .

Vertebrates unlike invertebrates, have an internal bony skeleton however invertebrates have exo-skeleton. This exo- skelton might constitute a useful target site for insecticidal cemicals (Gunnar et al, 1974). The special vulnerability of the insect cuticle is associated with the need to moult. Most of the cemicals that act to kill insects by affecting the cuticle do so by compromising the insects ability to moult. It might be a good target for the development of selective insecticides (Landureau and Grellet, 1975).

Most conventional insecticides are neuro-toxicant in their action, However, a number of agents are known or to have their primary effects on the cuticle as inert dust (Chen and Mayer, 1985), . Also IGR's are commonly disrupt moulting process. Certain chemical insecticides as acylurea acts by preventing synthesis and deposition of normal cuticle during the preparatory period of the moult – cycle that precede ecdysis(Deul et al ,1978) .

MATERIALS AND METHODS

Chitinase was prepared from its sources as reported by Deul *et al* (1978) where 3.3 g tissue was stored at 0 - 2 °C the remainder as homogenized in 10^{-3}M Cleland's reagent (dithiotheritol), DTT) (v/w =2). The homogenate was centrifuged for 15 min at 12,000g. An equal volume of saturated ammonium sulfate solution was slowly added to the supernatant. After stirring for 1hr, the suspension was centrifuged for 10 min at 10,000g. The precipitate was washed with half- sturated ammonium sulfate solution and was recentrifuged, after which it was suspended in a small volume of water, followed by dialysis against running tap water for 20 hr. Any occasional precipitate was removed by centrifugation and was discarded as it proved to be enzymatically inactive. After dialysis, water was added to the original ratio (v/w =2). All manipulations were carried out at 0 – 2 °C. Enzyme assay and measurement of N-acetylamino sugars. Chitinase were determined as reported by Reissig, *et al.* (1955) or mentioned and described by Pegg and Young (1982) using sodium acetate buffer instead of Tris-HCl buffer and wave-leangth 416 nm was used instead of 544 nm. 25 ul of chitin (20mg/ml), 100 ul of enzyme prep and 225 ul of sodium acetate buffer, pH 4.0 in total volume 350 ul. The enzyme substrate mixture was incubated at 35 °C for 60 min,then the reaction was stopped by adding 100 ul of 0.8 M borate buffer pH 10.0 followed by determination of N-acetylglucosamine by the method of Reissig, *et al.* (1955). By adding 1.5 ml of DMAB reagent. The samples were placed in a shaker water bath at 35°C for 20 min the samples were measured spectrophotometerically at wavelength 416 nm.

In the inhibition studies, of chitinase activity, 100 ul of the enzyme preparation was incubated with 10 ul of the inhibitor for 30 min, the enzyme-inhibitor mixture was used to measure the remaining activity. The percent in vitro inhibition was calculated using the following formula:

$$\% \text{ in vitro inhibition} = \frac{y - v_i}{v} \times 100$$

where : -

V is the specifie activty without inhbitor

Vi is the specifie activity in presence of inhibitor

RESULTS

A series of sodium acetate buffer solutions with pH values of 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, and 7 respectively were prepared. These solutions were used to measure the chitinase activity at different pH values. Table (1) shows that pH 4.0 ,is an optimum pH values for chitinase activity for extracted samples from Musca domestica , Periplaneta americana, Spodoptera littoralis, Culex pipiens. Which were similar for chitinase activity prepared from Trichoderma harzianum by Ulhoa and John, 1992.

To study the optimum time course of the chitinase activity, the enzymatic reaction was carried out at 15, 30, 45, 60, 75, 90, and 150 minutes as a time periods after adding the substrate solution.. The amount of produced N.AGA was determined at each of these intervals using Andrew et al method(1982). Table(1),represents the time course of the hydralysis of chitin in the persence of chitinase from different insect sources during this study.The results indicated that the hydrolysis of chitin was almost linear up to 60 minutes . Pegg and Young (1982) found that the time-course for chitin hydrolysis by chitinase was linear until 3 hr this difference may be attributed to the different sources of chitinase which were used or differences in the conditions of the enzymatic reaction.

The chitinase activity was measured at different temperatures. The temperature tested were 5, 15, 25, 35, and 45 ^{o}C. The activities of chitinase were calculated for different sources under the study . It is quite clear that the rate of chitinase-catalysed reaction reaches a maximum with increasing temperature. The optimum temperature, at which the maximum velocity occurs, was about 35^{o}C. Alain and Roby (1982) suggested that the optimum temperature for the melon seedlings chitinase was 37 ^{o}C. While the optimum

temperature for Trichoderma harzianum chitinase was 40 °C, as was recorded by Ulhoa and John (1992).

The relationship between the chitinase activity and the protein contents of different sources preparation was tested. The rate of chitin hydrolysis was measured at different protein concentrations. Results indicated that the chitin intial hydrolysis rate was directly proprotional to the protein concentration. An increase in activity of the chitinase was more evident within a range of 20-40 ug protein of Musca and Periplaneta chitinase and was 10-20 ug protein for Spodoptera and Culex chitinase.

Table (1) Optimum conditions for chitinase enzyme activity ,extracted from different sources

	Musca domestica	*Periplaneta americana*	*Spodoptera littoralis*	*Culex pipiens*
PH	4.0	4.0	4.0	4.0
Time	80	80	80	80
Temp.	35	35	35	35
Protein	32	28	10	12

The optimum conditions for maximum chitinase activity for the four different sources were at pH 4.0 and using 35 °C as a reaction temperature. The enzyme substrate mixtures were incubated for 60 min. in presence of optimum enzyme protein concentration for each source.

The effect of BSA on the chitinase activity was examined at concentrations range from 0.2 to 10 mg protein/ml in the reaction mixture. Chitinase activity was enhanced by the addition of BSA, maximum activity occurring at a concentration of 10 mg/ml BSA in the reaction mixture for the all chitinase sources. Similar results were reported by Pegg and Young (1982) ., using BSA in their studies of the effect of BSA on chitinase activity extracted from Verticillium albo-atrum. The effect of different concentrations

of Mg^{+2} , Ca^{+2} and Mn^{+2} on chitinase activity . No evidence for a requirement of metal ions (Mg^{+2}, Ca^{+2} or Mn^{+2}) was found, although activity was enhanced slightly. So, the chitinase has no cation requirements for activity. These results are parallel with that suggested by Andrew et al (1982) in their studies on purification and characterization of chitinase from the stable fly. Their data indicated that chitinase has no cation requirements for activity. , However Maria and Stinson (1981) found that the presence of Ca^{+2} gives high chitinase activity for larvae of Drosophila melanogaster.

Table (2) : Chitinase specific activities from different sources.

	M. Domestica	*P. americana*	*S. Littoralis*	*C. Pipiens*
Specific activity + SD (λ_{max} 416 mg^{-1} protein hr^{-1})	10.36 +0.32	21.72 +3.40	32.13 +1.20	22.59 +0.20
Diuron				
Km mg	0.006	0.005	0.004	0.002
Vmax mg	4.4	4.5	3.0	4.6
I50 uM	37.0	30.0	40.0	60.0
Ki uM	9.0	10.0	15	30.0
Chloropropham				
Km mg	0.002	0.003	0.004	0.002
Vmax mg	4.4	4.2	3.1	4.6
I50 uM	510	460	370	100
Ki uM	17	20	10	22

In- vitro inhibition of chitinase activity :

The chitinase enzyme activity was found to be higher in S. littoralis than C. pipiens, P. americana, and M. domestica. The most sensitive assay

for chitinase is a radiometric method by (Cabib, 1987) and that used in this study is a modified version of the method described by Reissig, et al. (1955). As discussed by Barrett-Bee and Hamilton (1984), the state of sub-division of chitin particles can influence reaction velocity. In addition. the specific activities were ranged from 10 to 32 / $_{max}$ 416 mg-1 protein hr^{-1} depending on insect stage, if it is immature or mature, Table (2). It is clear that the tested insect extracts were capable of degrading a chitin substrate which had been exposed to such mild conditions during preparation that there is good reason to believe it was relatively pure native chitin. The determination of chitinase activity in the various extracts of adult and in the pri of mature insects raises the question of the function of this enzyme in adult insects.

The I_{50} values of diuron and cloropropham against enzyme extracted from different sources as shown in Table (2), indicate that diuron and chloropropham are potent inhibitors of chitinase . The effect of tested compounds on some enzyme constants such as K_m value in presence of diuron or chloropropham shows as a potent competitive inhibitors in vitro for chitinase from all sources. K_i values were 15,10,9 and 30 uM of diuron and for chloropropham were 10,20, 17 and 22 uM against *S. littoralis, P.americana, M.domestica* and *C.pipiens* chitinase ,respectively.

Chitinase enzyme extracted from *Spodoptera littoralis* larvae as a source was the most sensitive to chloropropham with I50 3.7 $X10^{-5}$ M. Also chloropropham shows a competitive inhibition potency. It is clear from these results that the inibition constant values varies considerably from source to another source enzyme.

Propanil is increasing citinase activity in a concentration depandant manner, which is due to the different in structure configuration of propanil from diuron and chloropropham.

On the other hand the percent of chitinase inhibition by deltametrin, methocarb, aldicarb, paraoxon and DNOC were 21,20,18,17 and 15 respectively.

Propanil is increasing citinase activity in a concentration depandant manner, which is due to the difference in structure shape of propanil from diuron and chloropropham.

Effect of Chitinase inhibitor compounds on the biology of treated insects.

Using fifty adults of housefly insects M. domestica treated wit the I_{50} concentration of cloropropham, diuron, phenylurea, propanil and urea compounds. Reared under normal condition and calculate the different biological parameters during two successive generations.

Table (3): The effect on egg production of two successive generation produced after treatment of adults of M.domestica with tested compounds.

Comp.	*No of adult*	*Weight of egg (mg)*	*Days of laying*	*Time of Hatch.*	*Pupae produced*	*% pupation*	*Adult emerged*
Control							
F1	*50*	*207.6*	20	8,25	*2187*	*97.2*	*2117*
F2	*50*	*212.7*	21	9.6	*2200*	*98.0*	*2150*
Diuron							
F1	*50*	*70.7*	13	17.0	*612*	*79.0*	*478*
F2	*50*	*92.4*	15	15.0	*819*	*82.7*	*677*
Chloropropham							
F1	*50*	*92.1*	15	13.14	*652*	*80.9*	*524*
F2	*50*	*104.0*	16	11.0	*940*	*86.3*	*811*
Propanil F1	*50*	*197.9*	17	8.3	*2170*	*97.6*	*2117*
F2	*50*	*200.0*	18	9.0	*2275*	*98.2*	*2235*
Phenyl urea F1	*50*	*112.9*	17	10.40	*1120*	*92.1*	*1032*
F2	*50*	*122.4*	16	9,5	*1280*	*93.8*	*1200*
Urea							
F1	*50*	*139.9*	17	9.7	*1397*	*94.9*	*1325*
F2	*50*	*147.1*	17	7.8	*1460*	*97.3*	*1420*

Table (3), shows a prolongation of lapse time for all tested compounds except urea compared with mother colony. The most effective compound was diuron followed by cloropropham then phenyl urea and propanil.Also data shows shortining in days of egg laying . This agree with which found by Madore et. al , 1983. An intereasting feature was the pattern of te rate of decline in the percentage of pupation of the resulting progency of F_1 , F_2 generations following treatments with all tested compounds .This may be due to interference of tested compounds with the ability of insect instar to eat properly which is result from reduction of chitin biosynthesis (Abdel-Monem et al ,1980). Diuron was the most effective compound followed by chloropropam then phenylurea, urea and the least effective one was propanil on the biology of treated housefly strain.

The affect biological parameter depending mainly on the effect of these compounds on chitin synthesis inibition (Mayer et al. 1980). The studied compounds interferse cuticle deposition, apparently by inhibition of citin synthesis. This effect may due to the penetration of the treated compound to the peritropic membrane and passage through the gut epithelium. Perhaps chitinase treatment allows greater number of nucleocapsids to penetrate the damaged peritropic membrane,pass from the gut lumen to the hemocoel and infect susceptible cells without cycle of replication. (Shapiro et al ,1987 and Keiding et al, 1991).

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الملخص العربى

تأثير بعض المبيدات على انزيم الشيتينيز المستخلص من مصادر حشرية مختلفة

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تم استخلاص انزيم الشيتينيز من يرقات دودة ورق القطن و يرقات بعوض الكيولكس و الحشرة الكاملة للصرصار الامريكى و الحشرة الكاملة للذباب المنزلى. تم دراسة الظروف المثلى من pH ، حرارة تفاعل ، وزمن التفاعل و تركيز المحتوى البروتينى لنشاط الانزيم المستخلص من المصادر الحشرية المختلفة وذلك باستخدام مادة الشيتين النقى كمادة تفاعل للأنزيم وتم تقدير كل من Vmax , Km للأنزيم فى المصادر الحشرية المختلفة المستخلص منها.

تم تتبع اثر مجاميع المبيدات المختلفة على نشاط انزيم الشيتينيز للأفات المعاملة ، أظهرت قيمة تركيز I50 لمركب الديرون كانت ٤X١٠ $^{-٥}$ ، ٣X١٠ $^{-٥}$ ، ٣ر٧X١٠ $^{-٥}$ ، ٦X١٠ $^{-٥}$ مولر للأنزيم المستخلص من يرقات دودة ورق القطن ، الحشرة الكاملة للصرصار الامريكى ، الحشرة الكاملة للذباب المنزلى ويرقات البعوض على التوالى. بينما كانت القدرة التثبيطية لمركب الكلوربروفام ٧ر٣X١٠ $^{-٥}$ ، ٦ر٤X١٠ $^{-٥}$ ، ١ر٥X١٠ $^{-٥}$ ، ١X١٠ $^{-٤}$ مولر على التوالى لنفس المصادر الحشرية. بينما عند استخدام مركب البروبانيل عمل على زيادة نشاط الانزيم المستخلص من المصادر المختلفة عن مثيلاتها فى الكنترول.

عند اختبار تأثير مركبات الديلتاميثرين، ميثوكارب، الديكارب، باراكسون، DNOC كانت النسبة المئوية لتثبيط نشاط الانزيم٢١، ٢٠، ١٨، ١٧، ١٥ % على التوالى على الانزيم المستخلص من يرقات دودة ورق القطن.

. يوضح البحث اهمية دراسة تداخل المبيدات المستخدمة لمكافحة الافات المختلفة على محصول ما فى الحقل مع بعض الاهداف الحيوية الاخرى داخل الكائن المعامل مما يؤثر على نشاط وكفاءة الحشرة فى عمليات الانسلاخ والتطور وعدد الاجيال النشطة للحشرة مما يوضع فى الاعتبار عند وضع برنامج مكافحة متكامل لافات محصول ما.