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RESEARCH ARTICLE

Changes in enzyme activities in *Agrotis ipsilon* (Lepidoptera, Noctuidae) as a response to entomopathogenic nematode infection

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Abstract

In present study changes in acetylcholinesterase (AChE), carboxylesterase (CarE), phenoloxidase (PO), protease, amylase, invertase and trehalase enzyme activities in 4th larval instar of *Agrotis ipsilon* under entomopathogenic nematode infection was investigated. The nematode infection reduces the digestive enzymes activity (amylase, trehalase and inveratse). The inveratse activity fluctuate in *Heterorhabditis zealandica* infected larvae while *Steinernema abbasi* infected larvae show an increase in inveratse activity until 24 hrs post- infection then the activity was decreased compared with the control. Significant increase in AChE activity during the first 8 hrs of nematode infection was observed. At 40 hrs post-infection, *H. zealandica* infected larvae exhibited higher AChE activity compared with *S. abbasi* infected and control larvae. Inhibition of AChE in *S. abbasi* infected larvae results in hyperactivities and consequently paralysis and larval death. PO activity decreased in the nematode infected larvae compared with the control and the reduction was remarkable in *H.* zealandica infected larvae. The CarE activity was increased by the time increase in *S. abbasi* infected larvae, reflecting the overactive stress response to the nematode infection. The protease activity was decreased after 16 hrs post-infection with *H. zealandica* and *S. abbasi* compared to the control. The observed decreases in protease enzyme activity is expected due to the insect body protein digestion caused by the symbiotic bacteria 16 hours post-infection while the increase in AChE and CarE activity after 16 hrs post-infection is due to the toxic substances released by the bacteria in the hemocoel. Changes in enzyme activities under nematode infection indicate the overreact response to the nematode infection.

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INTRODUCTION

Agrotis ipsilon (Hufnagel) (Lepidoptera, Noctuidae) is more commonly known as the black or greasy cutworm. It is a polyphagous serious pest of different economic crop plants worldwide (Rings and Johnson, 1975). The larval stages can cause severe damage to golf courses as well as vegetables and field corps (Hong and Williamson, 2004).

Entomopathogenic nematodes (*Heterorhabditidae* and *Steinernematidae*) over an environmentally safe and IPM compatible alternative to chemical insecticides for the control of soil inhabiting insects (Kaya and Gayugler, 1993; Grewal *et al.,* 2005) have been applied. When the nematode was applied under conducive conditions, it has been as

effective as chemical insecticides against *Popillia japonica* larvae and white grubs (Georgis and Gaugler, 1991, Ibrahim 2005, and Ibrahim 2010).

Entomopathogenic nematode from the genus *Steinernematidae* and *Heterorhabditidae* was characterized by a symbiotic association with bacteria of the genera *Xenorhabdus* and *Photorhabdus,* respectively. The bacteria are contained in the intestine of the free- living infective juveniles (IJS) of these nematodes, which are capable of seeking out hosts (Ansari *et al.,* 2003). The IJS are enter the hemocoel of the insect and release the symbiotic bacteria (Kaya and Gaugler, 1993) that multiply in the hemolymph causing insect death within 48-72hr and establishing conditions for nematode development in the cadaver by providing nutrients (Nickel and Welch, 1984) , they also initially prevent the growth of other microorganism (Hoffmann 2003). Since the hemolymph is the main site of action, biochemical changes in its components are expected. ELBishry and Eid (1992) reported that hemolymph protein of *Spodoptera littoralis* sixth instar larvae is markedly reduced after 30 hrs of the infection with *Steinernema carpocapsae***.** Enzyme markers have been found to be especially suitable (Patnaik and Datta, 1995).

The physiological information of the entomopathogenic nematode pathogenicity on soil born insects are lacked. This study aimed to acquire knowledge on the activity changes in some immunity enzymes such as phenoloxidase, acetylcholinesterase and carboxylesterase, in addition to digestive enzyme such as protease which is essential for degradation of hemolymph proteins and carbohydrate hydrolyzing enzymes (amylase, trehalase and invertase) in *Agrotis ipsilon* infected with EPNs. We investigated the activity changes of these enzymes in the 4th larval instar of *Agrotis ipsilon* infected with two nematode strains (*H. zealandica* and *S. abbasi*) and control larvae as well. The data indicate that EPNs infection causes changes in immunity system and digestive responsive enzyme activities in a time dependent manner.

Material and methods

Black cutworm culture:

Agrotis ipsilon used in this study was obtained from the Department of Crop Pests, Plant Protection Research Institute, ARC, Dokki, Giza-Egypt. The culture was reared under laboratory conditions (28 ± 2 °C and 65 – 70 % R.H). The culture was provided with castor leaves (*Ricinus communis)* as a source of food inside glass jars with saw dust layer in the bottom to absorb humidity and covered with muslin fixed with a rubber band until the pupation. The newly emerged moths were mated within the large glass jars provided with a cotton piece soaked in 10% sugar solution as a feeding source.

Entomopathogenic nematodes:

Two entomopathogenic nematode strains namely *Heterorhabditis zealandica* and *Steinernema abbasi* were cultured in the last instar larvae of the greater wax moth (*Galleria mellonella* L.) according to Dutky *et al.,* (1964). The emerging infective juveniles (IJs) were harvested from White traps and stored in distilled water at 15°C and 4°C for the nematodes belonging to the genus *Heterorhabditis* and *Steinernema*, respectively

Nematode infection and sampling:

The fourth larval instar of *Agrotis ipsilon* was subjected to nematode infection under laboratory condition. The nematode strains *Heterorhabditis zealandica* and *S. Abbasi* was used at the dose of fifteen infective juveniles per larva. There was ten replication per treatment each contains 10 insects. Samples were collected for enzyme assay at 8, 16, 24, 32 and 40 hours post infection.

Enzymes preparation:

Enzymes were prepared by homogenization of 10 larvae in 40 mL ice-cold phosphate buffer (0.05 M, pH 7.0) by using an ultrasonic homogenizer at 30 W, 10 s. After centrifuging for 30 min (4°C, 10000×g), the final supernatant was used as enzyme solution. Protein content of enzyme solution was measured according to the method of Bradford (1976). All chemicals used were analytical grade and were obtained from Sigma.

Determination of enzyme activities:

All experiments were replicated three times each with ten larvae. Assays also were performed as three technical replicates per preparation for determination of phenoloxidase (PO), acetylcholinesterase (AChE), carboxylesterase (CarE), protease , amylase, trehalase and invertase activities. Enzymes activities were assessed following standard methods described by Sugumaran and Nellaiappan (2000), Ellman et al. (1961), van Asperen (1962), Tahoun and Abdel Ghaffar (1986), and Ishaya and Swirski (1976), respectively.

Statistical analysis:

Statistical analysis was performed using Analyze-it software (Analyze-it, Leeds, UK) in accordance with the method of Maxwell and Delany (1989). All results are expressed as mean ± SE. Statistical analysis of the data was performed using standard 1-way ANOVA. P value < 0.05 was considered to be statistically significant.

Results and Discussion

Acetylcholinesterase (AChE) is an important enzyme in the nervous system of insects, terminating nerve impulses by catalyzing the hydrolysis of neurotransmitter acetylcholine (ACh). Inhibition of AChE results in excessive accumulation of ACh, leading to hyperactivities and consequently paralysis and death (Soreq and Seidman, 2001). AChE is a target of organophosphorus and carbamate compounds in insects, which remain to be widely used pesticides around the world (Fournier and Mutero, 1994).

Entomopathogenic gram-negative bacteria produce toxins. Members in the Enterobacteriaceae such as *Photorhabdus* and *Xenorhabdus* spp. produce insecticidal toxins with oral toxicity similar to that of Bt (*Bacillus thuringiensis*) toxins (Anaïs and Patricia, 2014). These toxin complexes (Tc's) are large orally active toxins that are displayed on the outer surface of the bacterium. Pir proteins are *Photorhabdus* toxins that have been identified to have hemolymph (Ffrench *et al*., 2007) and oral (Blackburn *et al*., 2006) toxicity. According to Anaïs and Patricia (2014), the binding and destructive effects on neural tissue could be a major factor in toxicity when *Photorhabdus* are injected into susceptible insects. Leptinotarsin is a neurotoxin that stimulates release of acetylcholine at the presynaptic nerve terminal (Yoshino *et al*., 1980 and McClure *et al*., 1980).

Agrotis ipsilon 4th larval instar infected with *Heterorhabditis zealandica* and *Steinernema abbasi* were randomly selected from each group, at the time-point of 0, 8, 16, 24, 32 and 40 hrs post-infection respectively. Non treated larvae were used as a control and were sampled at the same time. Enzymes were isolated from the infected and control insect larvae. The results indicate that infection of *Agrotis ipsilon* with two entomopathogenic nematode strains for more than 8 hrs resulted in a significant increase in AchE activity. At 40 hrs post-infection, *H. zealandica* infected larvae exhibited higher AchE activity compared with *S. abbasi* infected and the control. The observed decreases in AChE activity at16 hrs post-infection compared with 8 hrs indicates that EPNs infection may consume too much AChE (Fig.1). The increase in AChE activity in *Agrotis ipsilon* larvae infected with *H. zealandica* may be an over reactive response to EPNs infection, which would eliminate much more ACh, consequently resulting in paralysis and death as well. This results are in agreement with the results reported by Soreq and Seidman, 2001 and with the results reported by Han-dong *et al*., (2013) who found that, the AChE activity of *G. mellonella* larvae after infecting with *H. beicherriana* was significantly increased compared with the control.

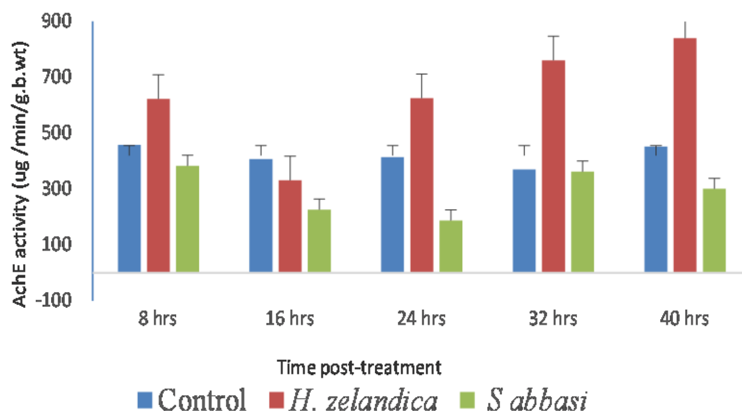

Fig1. Acetylcholinesterase activity in 4th larval instar of *Agrotis ipsilon* infected with EPNs (15 IJs/larva). Data are means ± SE

Carboxylesterase (CarE) known as the primary metabolic and hydrolyzing enzymes that cleave ester bonds (Oakeshott *et al.*, 2005).This detoxifying enzyme play an important role in insecticide resistance and have been associated with resistance to several insecticide classes in many insects (Ranson *et al.,* 2002 and de Carvalho *et al.,* 2006). Due to its role in insecticide metabolism, the measurement of carboxylesterase activity is used as a biochemical indicator of insecticide resistance in many insect species.

The results presented in Fig.(2) indicate that, the activities of CarE was increased in a time-dependent manner in *Agrotis ipsilon* larvae infected with *S. abbasi*, which may be the overactive stress response to the nematode infection. At 40 hrs post-infection, larvae infected with *S. abbasi* exhibited higher CarE activity compared with those infected with *H. zealandica* and the control (Fig. 2). This data indicate that the larvae increase the CarE

activity in their cell in order to overcome the toxic protein released by *Xenorhabdus* bacteria in the hemocoel. The decreases in CarE activity in the larvae infected with *H. zealandica* indicated that the insect immunity system is distributed so it cannot resist the toxic substances released by *Photorhabdus* bacteria in the hemocoel.

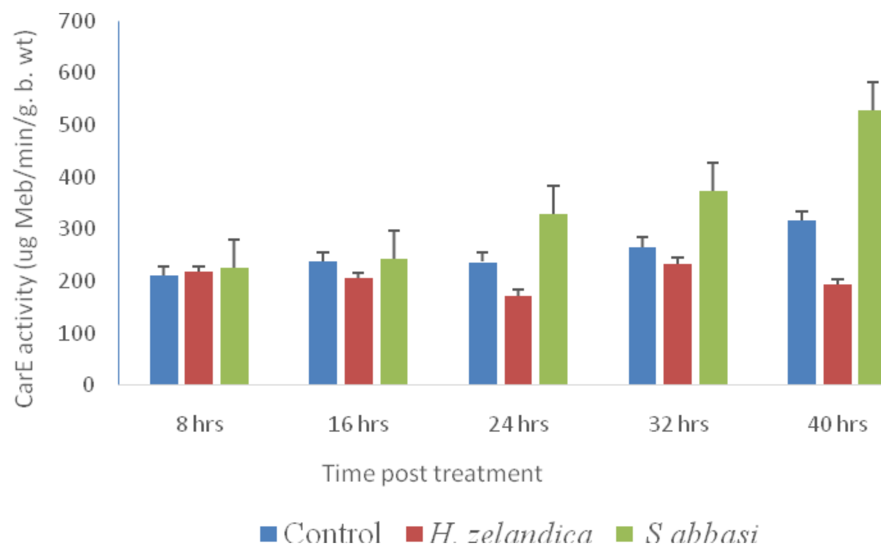

Fig2. Carboxylesterase activity in 4th larval instar of *Agrotis ipsilon* infected with EPNs (15 IJs/larva). Data are means ± SE

In Invertebrate immunology a complex array of host defenses have been reported which include phagocytosis, melanization (i.e., synthesis and deposition of melanin around the pathogen), (Bulet *et al.,* 2004; Nappi and Christensen, 2005). The cuticular phenoloxidase (PO) is a melanizing enzyme at the wound site which limits infection; PO in hemolymph is responsible for melanotic immune responses (Pham and Schneider, 2008). The PO immune responses occur immediately against invading microbes in insects (Castillo *et al.,* 2011). PO is a key component in the immune system of insects and the main role of PO in melanogenesis is converting phenols to quinones, which subsequently polymerize to form melanin (González-Santoyo and Córdoba-Aguilar, 2012).

Ebrahimi., *et al* (2014) reported that, PO activity of nematode-injected *Leptinotarsa decemlineata* has increased in the hemocoel in a nematode dose-dependent manner, increasing nematode concentration led to increased PO activity which was coincident with the appearance of symbiotic bacteria in the hemolymph of the insects .

Based on the data presented in Fig. (3) PO activity decreased in the nematode infected black cutworm larvae compared with the control, the reduction was much higher at the *H. zealandica* infected larvae may be due to the highly toxic compounds released by the *Photorhabdus* bacteria which induce PO inactivation in the body of *Agrotis ipsilon* larvae.

According to the data reported by Balasubramanian *et al* (2009) *S. carpocapsae*-secreted chymotrypsin proteases that can inhibit prophenoloxidase and prevent encapsulation in *G. mellonella*. The nematode species *Steinernema feltiae* induces prophenoloxidase inactivation in *G. mellonella* larvae by down-regulating the prophenoloxidase pathway (Brivio *et al* 2002). These results agreed and support our results in which *S. abbasi* induces phenoloxidase inactivation in *Agrotis ipsilon* larvae (Fig.3).

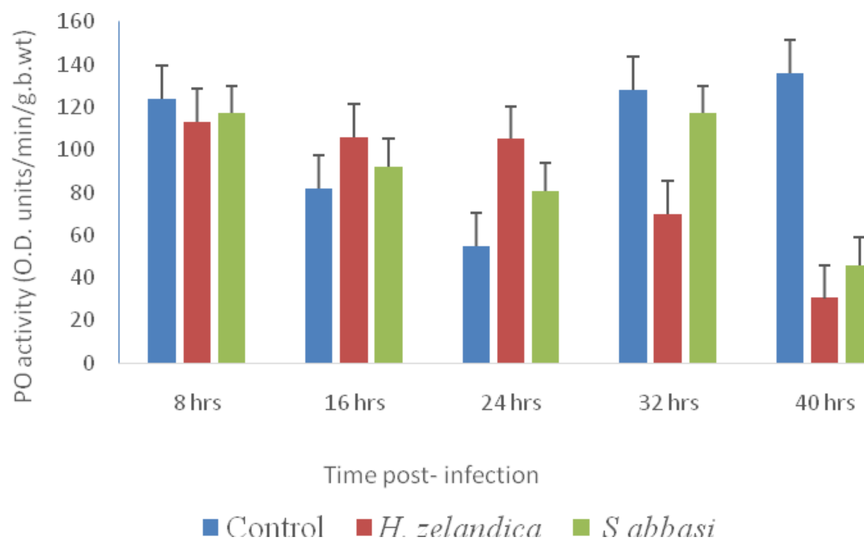

Fig3. Phenoloxidase(PO) activity in 4th larval instar of *Agrotis ipsilon* infected with EPNs (15 IJs/larva). Data are means ± SE

Changes in insect digestive enzymes under EPNs infection:

Changes in enzymatic activity during the progress of pathogen play an important role in understanding the interaction between the host and pathogen as a part of a survival strategy. In the present study changes in protease, amylase, invertase and trehalase activities during the progress of Entomopathogenic nematodes in *Agrotis ipsilon* larvae at 8, 16, 24, 32 and 40 hours post-infection was determined. The data indicate that, the protease activity decreased after 16 hrs of infection with *H. zealandica* and *S. abbasi* infected larvae compared to the control (Fig.4). The observed decreases in protease enzyme activity could be due to the symbiotic bacteria start to digest the proteins of the insect body 16 hours post infection while the increase in AChE and CarE activity after 16 hrs post-infection is due the toxic substances released by the bacterial blood (Fig1 and 2 respectively)..

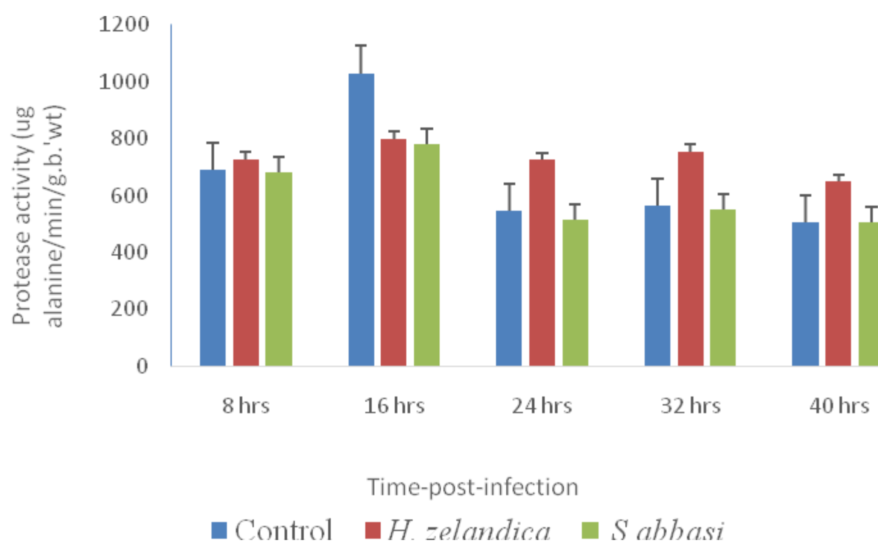

Fig 4. Protease activity in 4th larval instar of *Agrotis ipsilon* infected with EPNs (15 IJs/larva). Data are means ± SE

Amylase activity is decreased with increasing the time from 8 to 40 hrs post-infection (Fig5-A). The inveratse activity fluctuate in the *H. zealandica* infected larvae while *S. abbasi* infected larvae show an increase in inveratse

activity until 24 hrs post infection then the activity was decreased compared to the control (Fig5-B). The data indicate that 8 hrs post infection an increase of trehalase activity was observed in *S. abbasi* infected larvae, then the activity was declined while the activity of *H. zealandica* infected larvae was lower than the control after the first 8 and 24 hours (Fig. 5-C). It appears that the energy demands are stepped up in the host in initial stage of infection, when the physiology of the host is altered to combat the disease as a natural response. The decrease in the trehalase activity in the nematodes infected larvae could be attributed to decreased metabolic capabilities of infected larvae. This was also interpreted as due to decreased hydrolysis of trehalase to release glucose molecules under drastic stress conditions and high energy demand (Hasegawa and Yamashita, 1970).

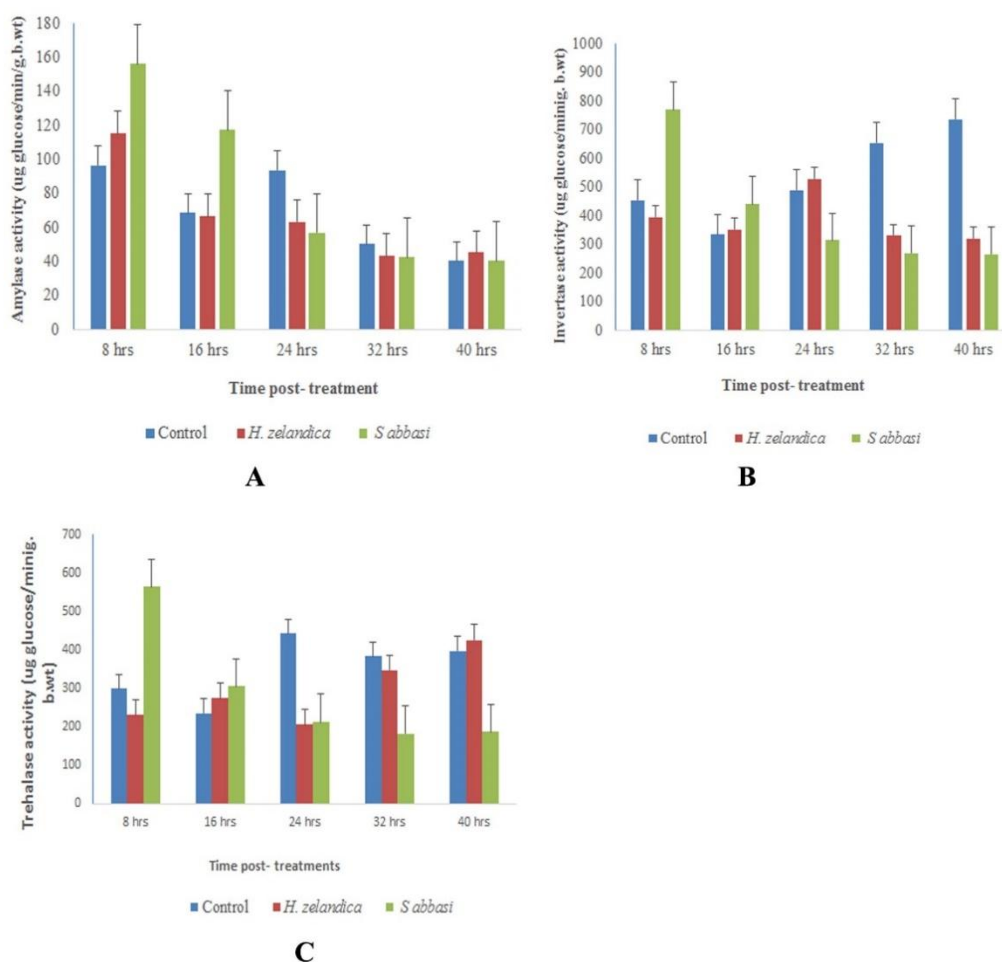

Fig 5. Digestive enzyme activities in 4th larval instar of *Agrotis ipsilon* infected with EPNs (15 IJs/larva). Data are means ± SE.

In conclusion, our data suggest that the activities of certain immunity related enzymes (PO, AchE and CarE) and the digestive enzyme (protease, amylase, trehalase and invertase) were changed in the 4th larval instar of *Agrotis ipsilon* by nematode infection in a time dependent manner. This may be the overactive stress response to the EPNs infection. This overreaction leads to death of the host insects.

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