0014-4754/93/490557-04$1.50 + 0.20  

# Hormetic concentrations of azadirachtin and isoesterase profiles in *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)

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*Received 26 May 1992; accepted 22 February 1993*

*Abstract.* Electrophoretic patterns of esterases from larvae of *Tribolium castaneum* fed on a diet which included hormetic concentrations of azadirachtin (viz. 0.001, 0.01 and 0.1 ppm) for 10 d were studied. The results showed a dose-dependent variation in the multiple molecular forms of the esterases. The variations, however, were not limited to the synthesis of new isoforms or the deletion of existing ones; there was also variation in their relative abundance.
*Key words.* Esterases; azadirachtin; hormetic concentrations; hormesis.

Potentially toxic substances at subinhibitory concentrations may also have stimulatory effects[1,2]. This phenomenon is called hormesis[2]. The hormetic effect of azadirachtin, a tetranortriterpenoid from *Azadirachta indica*, was reported for the first time in *Tribolium castaneum* by us. In this report it was shown that at 0.001 ppm to 0.1 ppm the larvae weighed more than the controls[3]. Azadirachtin, a potent insect growth inhibitor, is known to affect hormonal balance in insects[4] and hence its beneficial effects may be a consequence of favourable shift in hormonal balance, at subinhibitory concentrations. Esterases, in insects, have been implicated in reproductive behaviour[5], pheromone and hormone metabolism[6,7], digestion[8,9], neurotransmission[10], and the action of and resistance to insecticides particularly organophosphates (OPs)[11–13]. The mechanism of hormesis is still unknown, although several hypotheses have been proposed[1]. Hormesis has been suggested to be the consequence of transient or sustained overcorrections in response to low levels of inhibitory stimulus[1,14]. The present study, therefore, is an attempt at understanding, at the molecular level, the involvement of esterases in the hormetic action of azadirachtin on *Tribolium castaneum*.

## *Materials and methods*

A laboratory culture of *Tribolium castaneum* was maintained on a diet comprising white flour: white cornmeal: dried brewer's yeast (10:10:1.5) at 27 ± 1 °C and 60% RH. To obtain freshly hatched first instar larvae, eggs were obtained by infesting freshly sieved (mesh no. 40) diet with adults of both sexes. The eggs thus obtained were incubated at 30 °C to give freshly hatched first instar larvae.

Diet with different concentrations of azadirachtin was prepared by adding a known amount of azadirachtin (98% pure, a gift from Prof. H. Rembold, Max Planck Institut für Biochemie, Germany) from a stock solution. The carrier solvent used was AR grade acetone. Diet soaked in acetone alone served as the control. Treated and control diets were kept at 30 °C for 48 h to allow complete evaporation of the solvent.

Five freshly-hatched first instar larvae per replicate per concentration were allowed to feed on diet containing 0.001, 0.01 and 0.1 ppm azadirachtin or on the control diet for 10 d. For each treatment there were three replicate groups. This resulted in 15 ten-day-old larvae (III instar ~8 mg) per treatment after 10 d. These were separately homogenized in 3 ml of 40 mM phosphate buffer, pH 7.0. The homogenate was centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was used as the enzyme source.

Esterase activity was measured qualitatively using the polyacrylamide gel electrophoresis technique (PAGE). Disc electrophoresis was carried out on 10% acrylamide (pH 8.9)[15]. A void space of 0.5 to 1.0 ml at the top of each gel was left for loading the enzyme samples. Gels were polymerized using ammonium persulphate and residual persulphate was removed by pre-electrophoresis at 10 mA constant current for 30 min. A sample of supernatant from the larval homogenate corresponding to 50–60 µg was applied to each gel in 40% sucrose. Electrophoresis was carried out at 4 °C with 2 mA current/gel using 25 mM Tris and 192 mM glycine (pH 8.3) as the running buffer and bromophenol blue as the tracking dye. The run was terminated when the tracking dye band reached a point about 5 mm short of the lower end of the gel.

After electrophoresis, esterase bands were visualized by incubating the gels for 30–40 min in the dark in 100 mM phosphate buffer, pH 6.5, 0.02% 1-naphthyl acetate and 0.02% Fast Blue RR as a diazo coupler[16]. The gels were fixed in 7% acetic acid after the appearance of the bands. The gels were scanned at 540 nm on a Kontron Uvikon 860 spectrophotometer.

Relative mobility (Rm) of each band was calculated by the formula:

$$Rm = \frac{\text{distance travelled by isoenzyme band}}{\text{distance travelled by the dye front}}$$

*Results*

Perusal of the figure reveals the presence of five isoesterases in the control larvae fed on untreated diet. Isoesterases $E_1$, $E_3$, $E_5$, $E_{11}$ and $E_{15}$ have relative mobilities (Rm) of 0.08, 0.15, 0.20, 0.50 and 0.73 respectively (table 1), and a relative abundance of medium, low, high, very high and low, respectively (table 2). The isoesterases were distributed in three zones of activity, namely Est I, Est II and Est III (fig.). The larval population fed on a diet with 0.001 ppm azadirachtin did not show any deletion or disappearance of esterase isoforms in any of the three zones of activity, as compared to the control larvae fed on the untreated diet. The relative abundance of isoesterases $E_3$ and $E_{15}$ showed a definite increase over that in the control (fig.). Also, three new isoesterases not seen in control larvae were detected in the Est II zone; $E_8$, $E_{10}$ and $E_{12}$ (Rm 0.39, 0.45 and 0.53 respectively). All three had a very high relative abundance (tables 1 and 2).

In the case of larvae fed on the 0.01 ppm azadirachtin diet, two new slow-migrating isoesterases, $E_6$ and $E_7$, both of medium abundance (Rm 0.23 and 0.26 respectively) were found in zone Est I. Two new isoesterases, $E_8$ with medium and $E_{12}$ with very high abundance (Rm 0.39 and 0.53 respectively) appear in the Est II zone (tables 1 and 2). In the Est III zone one new isoesterase, $E_{14}$, was observed with very high abundance (Rm 0.71). None of the isoesterases seen in control larvae fed on untreated diet was deleted. The relative abundance of isoesterase $E_1$ and $E_5$ from the Est I zone declined marginally and that of $E_{15}$ from the Est III zone was substantially higher than in the control larvae. In larvae fed on a diet treated with 0.1 ppm azadirachtin six new isoesterases were present, and one was deleted. $E_2$ and $E_4$ (Rm 0.10 and 0.18 respectively), two slow-migrating isoesterases, were found in high abundance in zone Est I. Two new isoesterases migrating at medium speed, $E_8$ and $E_9$ (Rm 0.39 and 0.42 respectively), which were not detected in control larvae fed on untreated diet, were detected in the Est II zone in low abundance. Two fast-migrating isoesterases; $E_{13}$ (Rm 0.66) present in low abundance, and $E_{16}$ (Rm 0.75) in high abundance, were synthesized in zone Est III. Furthermore, the synthesis of the slow-migrating isoesterase $E_3$ (Rm 0.15) from the Est I zone was inhibited in the case of larvae fed on the diet with 0.1 ppm azadirachtin (table 1).

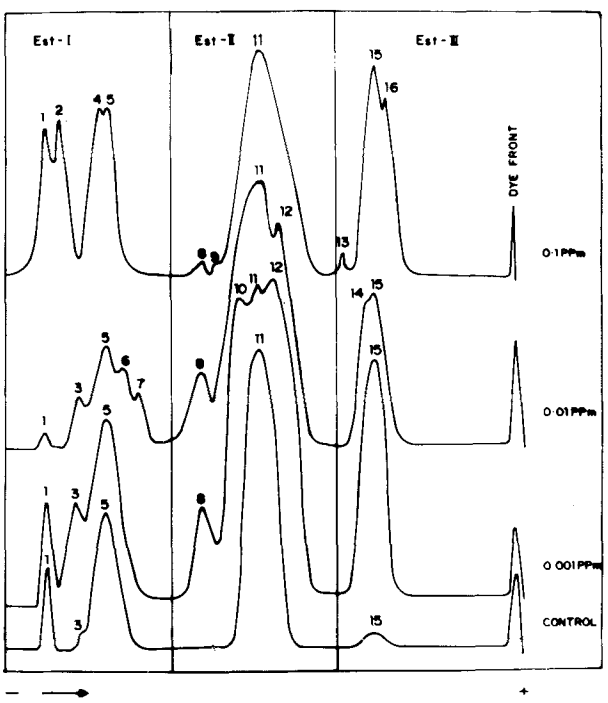


Figure. Esterase Isoenzyme profiles in *T. castaneum* in response to hormetic concentrations of azadirachtin (0.001, 0.01, 0.1 ppm). Est I: esterase activity zone I; Est II: esterase activity zone II; Est III: esterase activity zone III.

*Discussion*

In insects, esterases are known to detoxify injurious amounts of internal agents (e.g. hormones) or external substances (e.g. insecticides) by converting them into hydrophilic, excretable compounds. Increased activity of the enzymes, or the induction of multiple forms which are stereochemically adapted to the injurious agents, would render this kind of defence system very effective[17,18]. Our results suggest that new synthesis and/or repression of existing isoesterases could possibly be a contributing factor in the phenomenon of hormesis observed in *T. castaneum*.

Perusal of the figure reveals that induction and/or repression of esterase gene(s) function(s) could take place in response to hormetic concentrations of azadirachtin. New Est I zone isoesterases $E_6$ and $E_7$ (Rm 0.23 and 0.26 respectively) and $E_2$ and $E_4$ (Rm 0.10 and 0.18 respectively) were synthesized in larvae fed on diet containing 0.01 and 0.1 ppm azadirachtin respectively. This zone of activity was not, however, affected by the incorporation of 0.001 ppm azadirachtin in the diet. The synthesis of isoesterase $E_3$ (Rm 0.15) was not affected with incorporation of 0.001 and 0.01 ppm azadirachtin in the diet; however, at 0.1 ppm its expression was suppressed. This was the only case where suppression of an isoesterase was observed. Isoesterases $E_8$, $E_{10}$ and $E_{12}$ (Rm 0.39, 0.45 and 0.53), $E_8$, $E_{12}$ (Rm 0.39 and 0.53) and $E_8$ and $E_9$ (Rm 0.39 and 0.42) in the Est II zone were expressed in response to 0.001, 0.01 and 0.1 ppm azadirachtin treatment respectively. Among all the isoesterases synthesized in response to azadirachtin treatment, only $E_8$ in the Est II zone was induced at

Table 1. Selective expression and/or deletion of esterase isoforms* in *Tribolium castaneum***

| Treatment | Zones of activity Est I | | Est II | | Est III | |
|---|---|---|---|---|---|---|
| Control | $E_1$ (0.8)<br>$E_3$ (0.15)<br>$E_5$ (0.20) | | $E_{11}$ (0.50) | | $E_{15}$ (0.73) | |
| | New expression | Deletion | New expression | Deletion | New expression | Deletion |
| 0.001 ppm | - | - | $E_8$ (0.39)<br>$E_{10}$ (0.45)<br>$E_{12}$ (0.55) | - | - | - |
| 0.01 ppm | $E_6$ (0.23)<br>$E_7$ (0.26) | - | $E_8$ (0.39)<br>$E_{12}$(0.55) | - | $E_{14}$ (0.71) | |
| 0.1 ppm | $E_2$ (0.10)<br>$E_4$ (0.18) | $E_3$ (0.15) | $E_8$ (0.39)<br>$E_9$ (0.42) | - | $E_{13}$ (0.66)<br>$E_{16}$ (0.75) | - |

*E = esterase isoforms.
**Values in parenthesis refers to the $R_m$ values.

Table 2. Relative abundance of isoesterases* in *Tribolium castaneum*.

| Esterase isozyme numbers | Control | 0.001 ppm | 0.01 ppm | 0.1 ppm |
|---|---|---|---|---|
| Est I | | | | |
| $E_1$ | M | M | L | H |
| $E_2$ | - | - | - | H |
| $E_3$ | L | M | M | - |
| $E_4$ | - | - | - | H |
| $E_5$ | H | H | H | H |
| $E_6$ | - | - | M | - |
| $E_7$ | - | - | M | - |
| Est II | | | | |
| $E_8$ | - | M | M | L |
| $E_9$ | - | - | - | L |
| $E_{10}$ | - | I | - | - |
| $E_{11}$ | I | I | I | I |
| $E_{12}$ | - | I | I | - |
| Est III | | | | |
| $E_{13}$ | - | - | - | L |
| $E_{14}$ | - | - | I | - |
| $E_{15}$ | L | I | I | I |
| $E_{16}$ | - | - | - | I |

*L = low, M = medium, H = high, I = very high.

all the three hormetic concentrations of azadirachtin tested.

In zone Est III, no new isoesterase was induced in response to 0.001 ppm azadirachtin. However, $E_{14}$ (Rm 0.71) at 0.01 ppm, and $E_{13}$ and $E_{16}$ (Rm 0.66 and 0.75 respectively) at 0.1 ppm, were induced in this zone. The expression and abundance of the isoesterases $E_5$ and $E_{11}$ (Rm 0.20 and 0.50 respectively) was not affected in the larvae at any of the three hormetic levels of azadirachtin used. Whereas $E_{15}$ showed definite enhancement in level of abundance in the presence of hormetic concentrations of azadirachtin, $E_5$ and $E_{11}$ were not affected at all (fig.).

Our earlier work[3] has shown that larval weight and survival of larvae fed on hormetic concentrations of azadirachtin (viz. 0.001, 0.01 & 0.1 ppm) for 10 d was significantly higher than that of controls. Hence, it is suggested that hormetic concentrations of azadirachtin affect the synthesis or repression of esterases in *T. castaneum* larvae in a manner that will help them to grow and survive more efficiently. Since esterases have been implicated in hormone metabolism[7] and digestion[8,9], a model which may possibly explain such stimulation (as in case of the present study) is that the inhibitor may increase the activity of the polymorphic allosteric enzymes, and possibly of other protein systems. This could take place through selective gene(s) responsible for synthesis of the activation of the enzyme(s) which can detoxify the inhibitor. This activation could lead to the formation of several potentially highly active enzyme units (e.g. isoesterase $E_{15}$), as has been claimed in the case of insecticide potentiation[18]. It could equally well be the case in the present study that selective synthesis and/or deletion of esterase isoforms, in response to different levels of azadirachtin, contributed towards detoxification of the inhibitor molecules(s) or modulated the endogenous larval hormonal levels in a manner which is conducive to larval growth and survival.

It will be of interest to have more precise data on the origin of changes in isoesterase profiles, mainly by developing experiments on possible changes in tissue levels, since in insects esterase activities are different in gut, fat body and haemolymph.

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