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Plumbagin and azadirachtin deplete hemolymph ecdysteroid levels and alter the activity profiles of two lysosomal enzymes in the fat body of *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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Abstract. The profile of hemolymph ecdysteroid was studied in the gram pod borer, *Helicoverpa armigera*, during larval-pupal transformation. The changes closely correspond to the developmental events occurring at metamorphosis. Two insect growth regulators, plumbagin and azadirachtin, significantly depleted the content and altered the profile of ecdysteroids at crucial stages, when applied at ED_{50} doses. The activity profiles of two fat body lysosomal enzymes, acid phosphatase and β-galactosidase, were also significantly affected by the insect growth regulators. It is suggested that plumbagin and azadirachtin treatments primarily modify the ecdysteroid titer, which in turn leads to changes in lysosomal enzyme activity causing overt morphological abnormalities during the metamorphic molt.

INTRODUCTION

Growth and development in insects, which are punctuated by periods of molting, are regulated by the steroidal molting hormone, 20-hydroxyecdysone, and the sesquiterpenoid, juvenile hormone (Smith, 1985; Rees, 1995; Dhadialla et al., 1998). Endopterygotes endowed with complete metamorphosis are characterized by three distinct post-embryonic stages separated by two metamorphic molts, the larval-pupal and pupal-adult molts. Molting hormone triggers the molting events, whereas the character of the molt is dictated by juvenile hormone. Molting in the presence of a reduced titer or in the absence of juvenile hormone would result in larval-pupal or pupal-adult transformation, respectively (Gilbert & King, 1973; Smith, 1985). Imbalances in the level of the two morphogenetic hormones lead to abnormal forms i.e. prothetely or metathetely (Wigglesworth, 1972).

One of the striking features of holometabolous insects is the histolysis of larval organs during metamorphosis, which is necessary for cell remodelling. The break down of larval organs and cellular disintegration is mediated by hydrolytic enzymes which occur within membrane bound granules called secondary lysosomes. The various lysosomal enzymes involved in histolysis include acid phosphatase, β-galactosidase, α-galactosidase, α-glucosidase, α-mannosidase, β-glucosidase, β-N-acetyl glucosaminidase and β-glucuronidase. These lysosomal hydrolases are regulated directly or indirectly by the insect hormones that control post-embryonic development (Deloach & Mayer, 1979; Verkuil, 1980; Deloach et al., 1981). The activity of acid phosphatase, a marker enzyme of the lysosomal system, in the larval fat body of *Spodoptera litura* (Prasada Rao, 1990) and *Calliphora erythrocephala* (Verkuil, 1980) increases at the same time as the cessation of feeding (gut-purge) whereas β-galactosidase, a lysosomal glycosidase, participates in the pre-metamorphic lysosomal activity in the fat body of *C. erythrocephala,* and its activity increases significantly during the prepupal stage (Verkuil, 1980). This suggests ecdysteroid mediated changes in the activities of the two enzymes are occurring.

Identification of phytochemicals which mimic insect morphogenetic hormones or have growth regulating activity and synthesis of potent hormone agonists and antagonists in the recent past have led to their consideration as components of biorational approach to pest management. Two well known natural products, plumbagin from *Plumbago capensis* and azadirachtin from the *neem* tree *Azadirachta indica*, cause an anti-ecdysone effect and interfere with insect ecdysis (Sieber & Rembold, 1983; Gujar & Mehrotra, 1988; Joshi & Sehnal, 1989; Koolman, 1990; Marco et al., 1990; Singh & Bhathal, 1994). The present study was therefore undertaken to determine the effect of plumbagin and azadirachtin on the hemolymph ecdysteroid titer and the activities of two important lysosomal enzymes acid phosphatase and b-galactosidase, in the fat body of *Helicoverpa armigera* (Hubner), a polyphagous pest of economic importance.

MATERIAL AND METHODS

Chemicals

Plumbagin was obtained from Aldrich Chemical Company, USA and azadirachtin-A (99%) was a gift from Prof. H. Rembold, Max Planck Institute for Biochemistry, Munich, Germany. Radio-labelled ecdysone (NET-621, ecdysone α-[23,24-^{3}H (N)], specific activity 50–110 Ci/mmol) was purchased from American Radio Chemicals Corporation, USA. The ecdysteroid antiserum “2A” used in this study was produced by Prof. W.E. Bollenbacher, University of North Carolina, Chapel Hill and distributed by Prof. E.S. Chang, Bodega Marine Laboratory, Bodega Bay, California. 20-hydroxyecdysone, *p*-nitrophenyl phosphate, *p*-nitrophenyl β-D-galactopyranoside and *p*-nitrophenol were obtained from Sigma Chemical Company, USA. All other reagents were of analytical grade.

Insects

Helicoverpa armigera were reared on a semi-synthetic diet based on chickpea (Singh & Rembold, 1992) at 25 ± 1°C, 14L : 10D photoperiod and 60% r.h. Plumbagin was topically applied on the final instar larvae, 0–4 h after molting, at the ED_{50} dose of 100 $\mu g \cdot g^{-1}$ (Krishnayya & Rao, 1995). Azadirachtin, dissolved in a solution of ethanol and 0.7% NaCl (1 : 1), was injected (to avoid the antifeedant effect) into one-day old final instar larvae at the ED_{50} dose of 1.25 $\mu g \cdot g^{-1}$ (Mordue Luntz & Blackwell, 1993).

Radioimmunoassay (RIA)

Hemolymph samples (20 µl, 6–8 replications) were drawn at 6 or 12 h interval from plumbagin-treated, azadirachtin-treated and control larvae, by puncturing the base of a proleg. Samples were then transferred into clean, pre-chilled, eppendorf vials containing 200 µl of 70% methanol. All the RIA steps were completed in the same tube following the protocols of Borst & O'Connor (1972). The standard curve was developed under identical incubation conditions for 20-hydroxyecdysone in the range of 50–20,000 pg and the hemolymph hormone titers were determined by a regression equation.

Collection of fat body

Fat bodies were dissected out in a lepidopteran saline solution (Bindokas & Adams, 1988). Samples were drawn from final instar larvae at 30 h interval from plumbagin-treated, azadirachtin-treated and control larvae. After clearing off debris, the samples were frozen, freeze dried and stored at –70°C. Fat bodies pooled from three to four insects constituted one replicate and nine such replicates were collected for each stage of analysis. Samples collected at 180 and 210 h from the control insects represent pupal fat bodies.

Acid phosphatase assay

Lyophilized fat bodies were homogenized in 1% Triton X-100 (ice cold) and used as an enzyme source. Acid phosphatase activity was assayed in 100 mM acetate buffer (pH 5.5) containing 5 mM $MgCl_2$, 3 mM *p*-nitrophenyl phosphate and 0.1% Triton X-100 at 30°C for 30 min as previously described (Deloach & Mayer, 1979). The reaction was stopped by the addition of 300 µl of 6 M KOH. The controls were incubated similarly, but KOH was added at the beginning of each assay. After development of a yellow colour, the absorbance was read at 410 nm in a double-beam spectrophotometer (λ 3B, Perkin Elmer). *p*-nitrophenol was used as standard.

β-galactosidase assay

The assays were carried out in citrate buffer (0.2 M Na_2HPO_4-0.1 M citric acid, pH 3.6) containing 2.5 mM *p*-nitrophenyl β-D-galactopyranoside at 37°C for 30 min according to Conchie et al. (1959). The reaction was stopped by adding 400 µl of glycine-NaOH buffer (0.4 M glycine-6 M NaOH, pH 10.6). After development of the yellow colour formed by the released *p*-nitrophenol, the absorbance was read at 410 nm. The enzyme activities were expressed as mmol of *p*-nitrophenol released $h^{-1} \cdot g^{-1}$ tissue.

Protein content was determined by a modified version of Lowry's method (Dulley & Grieve, 1975) as the sample contained Triton X-100. The specific activities of enzymes were expressed as activity per mg protein.

Statistical analysis

The RIA data were subjected to analysis of variance (ANOVA) and the significance of differences among the means was determined by an "F" test. The mean response of the enzyme assays from the control and treatment trials were compared using a Student's t-test (Gomez & Gomez, 1984).

RESULTS

Ecdysteroid titer

The ecdysteroid profile shows significant variation in the treated *H. armigera* during larval-pupal transformation. In the penultimate larval instar, ecdysteroid titer was characterised by a significant increase to the level of about 163 ± 23 $ng \cdot ml^{-1}$ at 36 h after fourth instar, which significantly further increased to a peak of 227 ± 45 $ng \cdot ml^{-1}$ at 48 h. This peak was significant at a level of $P < 0.05$, relative to the titers of E + 24 and E + 30 (E = ecdysis to fourth / final instar). Only one significant peak of ecdysteroid titer was observed at the penultimate instar. There was a drastic reduction in the ecdysteroid titer prior to and after molting to the final instar (Fig. 1).

Four major ecdysteroid peaks were observed in the last larval instar. The first significant peak occurred at 84 h of the last instar, reaching a maximum of 1272 ± 69 $ng \cdot ml^{-1}$, followed by a small shoulder peak at 102 h attaining a

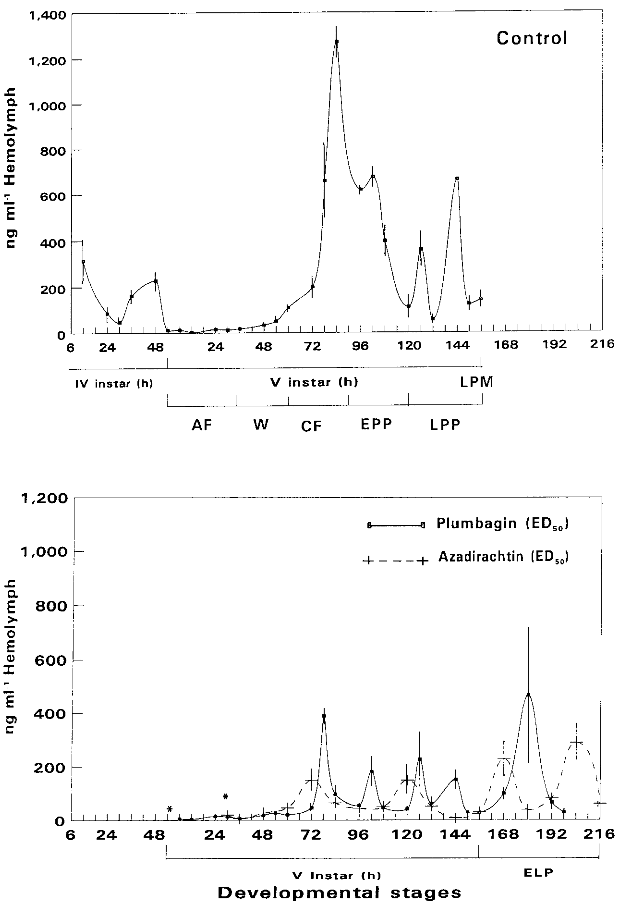

Fig. 1. Hemolymph 20-hydroxyecdysone titer of *H. armigera*. AF – active feeding; W – wandering; CF – cocoon formation; EPP – early pre-pupal; LPP – late pre-pupal; LPM – larval pupal molt; ELP – extended larval period. Vertical bars denote ± SD; * – time of application.

maximum of 678 ± 44 ng·ml^{-1}. These peaks were significantly higher than those noted upto E + 72 ($P < 0.001$). Following the shoulder peak, the ecdysteroid level dropped to 113 ± 53 ng·ml^{-1} at 120 h and then rose slightly, but significantly, to 361 ± 76 ng·ml^{-1} at 126 h. Peak 3 (126 h) was significantly higher than the peaks at E + 120 and E + 132 ($P < 0.001$). The last peak of RIA activity, 12 h before the metamorphic molt, was found to be 669 ± 0.1 ng·ml^{-1} at 144 h, which was significantly different from E + 120, E + 150 and E + 156 (all $P < 0.001$). Ecdysteroid titer dropped to a low of 145 ± 37 ng·ml^{-1} prior to the larval-pupal molt. The ecdysteroid titer thus dropped slightly after the larval-larval molt and then rose at different rates on at least four occasions, depending upon the stage of development, until the metamorphic molt.

The overall profile of molting hormone levels throughout the last instar of the plumbagin-treated insects showed lower ecdysteroid titers than the controls. No temporal delay in the appearance of the peak was evident and none of the four peaks noticed in the control were lost. There were five ecdysteroid titer peaks, observed at 78, 102, 126, 144 and 180 h. The fifth and the largest peak (468 ± 257 ng·ml^{-1}) was noted in the extended larval stage. None of the peaks were greater than 475 ng·ml^{-1}. A similar lower ecdysteroid titer was also observed in azadirachtin-injected insects compared to that of control. There were significant peaks of ecdysteroid titers observed at 72, 120, 168 and 204 h, two in the normal and two in the extended larval stage. None of the peaks were greater than 300 ng·ml^{-1}. In azadirachtin-treated insects, the latter three ecdysteroid peaks were delayed and had a reduced titer, whereas the first peak appeared earlier and also had a reduced hormone titer. None of the insects displayed any typical pre-metamorphic behaviours such as the cessation of feeding, gut-purge, wandering and cell formation for pupation up to 140 h. The larval-pupal molt was abnormal, and occurred 76 h later than expected, at 216 h.

Enzyme assay

Acid phosphatase

Fat body acid phosphatase activity during the larval-pupal transformation phase of *H. armigera* varied from 48.95 mmol at the larval active feeding stage to 195.21 mmol *p*-nitrophenol released h^{-1}·g^{-1} fat body at the pupal stage (Table 1). The specific activity of the enzyme also presents a similar trend and was lowest in the late pre-pupal stage. Two peaks in the enzyme activity are thus observed, one at the wandering stage of the larvae and the other immediately after the larval-pupal molt. This latter peak is relatively larger and broader than the former. Protein content in the fat body varied from 62.30 to 144.58 mg·g^{-1} during the last larval instar. It increased with age except at the early pre-pupal stage when it dropped to 60 mg·g^{-1}. A sharp increase in fat body protein content was observed in the late pre-pupal stage (112.54 mg·g^{-1}). It increased further in the pupal stage, where it reached as high as 144 mg·g^{-1}.

Fat body acid phosphatase activity in the plumbagin-treated insects varied between 31.14 to 82.72 mmol *p*-nitrophenol released h^{-1}·g^{-1} fat body. Mean enzyme activity, specific activity and protein content were significantly lower than the respective values for the control larvae. At 120 h the protein content was significantly higher, whereas acid phosphatase activity was reduced. It is pertinent to state that plumbagin-treated insects failed to undergo the larval-pupal transformation and had a prolonged larval life. The total acid phosphatase activity in the fat body of azadirachtin-treated insects was significantly reduced at all developmental stages of larval-pupal transformation. Changes in the fat body protein content were comparable to those of plumbagin-treated insects. The specific activity of the enzyme was greatest at 60 and 210 h. However, at 90 and 150 h the specific activity was on a par with that of the control.

β-galactosidase

Table 2 shows that fat body total enzyme activity was at a low level during the active feeding stage and the early pupal stage, and then increased very rapidly during the pupal stage. Thus, a major and broad peak in the activity of β-galactosidase was found commencing in the early pre-pupal stage and it remained high in the freshly molted pupae. Specific activity declined to the lowest levels during the wandering stage, but then increased to the highest activity levels during the pupal stage.

The total and specific activities of β-galactosidase were reduced significantly in the plumbagin-treated larvae at all stages of development. The reduction in total enzyme activity was appreciable during the last two stages of development (3–4 fold), when the plumbagin-treated insects failed to pupate normally. The total activity of β-galactosidase was lower in the azadirachtin-treated larvae when compared with the respective controls. Although specific activity was also reduced, it was on a par with the controls at 60 and 150 h. The differences noted in enzyme activities of the two controls (acetone topical and ethanol injection) were below 5–7%.

DISCUSSION

Remarkable changes in ecdysteroid titer are found throughout the life of an insect. Two critical peaks, the commitment peak and the pre-pupal peak prior to a metamorphic molt, reported by Dean et al. (1980), are also prominent in our study. Ecdysteroid levels are much higher and covered a broader period of time, as seen with the major peak that rose to 1,272 ng·ml^{-1} and persisted for over 36 h. A similar high titer (1,600 ng·ml^{-1}) was observed on the fourth day of *Heliothis virescens* final instar larvae (Barnby & Klocke, 1990). This peak is responsible for reprogramming of epidermal commitment from larval to pupal cuticle, and therefore for the well documented induction of gut-purge, dorsal vessel exposure, wandering behaviour and other accompanying prodromes of pupation (Truman & Riddiford, 1974; Karlson, 1980). The second peak, observed at 144 h, initiated apolysis and pharate pupal development which includes pupal cuticle secretion and successful larval-pupal transformation (Rid-

TABLE 1. Acid phosphatase activity in the fat body of plumbagin and azadirachtin-treated final instar *H. armigera*.

Age of last instar (physiological stage of control)	Plumbagin (ED_{50})						Azadirachtin (ED_{50})					
	mmol pNP released/h				Protein (mg/g)		mmol pNP released/h				Protein (mg/g)	
	/g tissue		/mg protein				/g tissue		/mg protein			
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
30 h (active feeding)	48.95±2.49	43.71*±2.42	0.79±0.05	0.74*±0.06	62.30±1.36	58.90*±1.82	48.95±2.49	48.95±2.49	0.79±0.05	0.79±0.05	62.30±1.36	62.30±1.36
60 h (wandering)	119.43±2.94	82.72*±3.48	1.48±0.04	1.31*±0.08	80.49±0.78	63.35*±1.25	123.72±3.01	72.15*±1.19	1.63±0.07	1.28*0±0.06	76.12±1.72	56.28*±2.04
90 h (cocoon formation)	108.25±3.88	66.97*±4.69	1.17±0.06	0.93*±0.09	92.76±1.83	72.07*±2.57	105.91±3.33	83.78*±2.68	1.22±0.06	1.17±0.03	86.82±1.86	71.34*±0.070
120 h (early pre-pupa)	64.04±4.38	46.23*±2.18	0.99±0.07	0.58*±0.04	64.66±1.44	79.90**±1.36	62.90±3.55	42.11*±1.58	1.02±0.05	0.51*±0.02	61.89±0.83	81.86**±1.58
150 h (late pre-pupa)	50.95±3.50	31.14*±4.24	0.45±0.02	0.35*±0.05	112.54±2.48	88.02*±1.24	48.19±4.00	32.11*±0.59	0.46±0.03	0.47±0.02	105.10±1.98	67.77*±1.81
180 h (pupa)	157.47±2.76	38.44*±0.43	1.20±0.02	0.49*±0.02	131.56±1.01	79.39*±2.38	150.57±4.72	55.97*±0.32	1.17±0.05	0.88*±0.02	129.39±1.77	63.98*±1.64
210 h (pupa)	195.21±2.41	57.29*±1.18	1.35±0.04	0.65*±0.01	144.58±2.20	88.86*±1.34	193.18±5.84	63.99*±1.14	1.28±0.04	0.84*±0.05	150.65±1.17	76.00*±3.12

Each value is a mean of 9 samples ± SD; values expressed are for freeze dried fat body; *p*NP = *p*-nitrophenol.
* Significantly lower (t = 0.05) than the corresponding control values.
** Significantly higher (t = 0.05) than the corresponding control values.

TABLE 2. β-galactosidase activity in the fat body of plumbagin and azadirachtin-treated final instar *H. armigera*.

Age of last instar (physiological stage of control)	Plumbagin (ED_{50})						Azadirachtin (ED_{50})					
	mmol pNP released/h				Protein (mg/g)		mmol pNP released/h				Protein (mg/g)	
	/g tissue		/mg protein				/g tissue		/mg protein			
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
30 h (active feeding)	1.94±0.14	0.99*±0.03	0.031±0.002	0.017*±0.001	62.30±1.36	58.90*±1.82	1.94±0.14	1.94±0.14	0.031±0.002	0.031±0.002	62.30±1.36	62.30±1.36
60 h (wandering)	1.70±0.07	0.50*±0.02	0.021±0.001	0.008*±0.003	80.49±0.78	63.35*±1.25	1.63±0.11	0.70*±0.03	0.021±0.002	0.012*±0.010	76.12±1.72	56.28*±2.04
90 h (cocoon formation)	4.48±0.03	2.80*±0.11	0.048±0.001	0.039*±0.006	92.76±1.83	72.07*±2.57	4.16±0.09	1.63*±0.06	0.048±0.002	0.023*±0.009	86.82±1.86	71.34*±0.070
120 h (early pre-pupa)	6.63±0.07	4.48*±0.19	0.103±0.002	0.056*±0.003	64.66±1.44	79.90**±1.36	6.21±0.14	3.38*±0.10	0.100±0.009	0.041*±0.008	61.89±0.83	81.86**±1.58
150 h (late pre-pupa)	10.6±0.12	7.86*±0.17	0.094±0.002	0.089*±0.002	112.54±2.48	88.02*±1.24	9.87±0.11	6.18*±0.07	0.094±0.008	0.091±0.004	105.10±1.98	67.77*±1.81
180 h (pupa)	29.09±0.13	6.71*±0.15	0.221±0.002	0.085*±0.004	131.56±1.01	79.39*±2.38	27.38±1.30	6.75*±0.05	0.212±0.013	0.106*±0.003	129.39±1.77	63.98*±1.64
210 h (pupa)	25.43±0.16	9.29*±0.08	0.175±0.001	0.105*±0.006	144.58±2.20	88.86*±1.34	22.58±1.23	6.22*±0.02	0.150±0.009	0.082*±0.004	150.65±1.17	76.00*±3.12

Each value is a mean of 9 samples ± SD; values expressed are for freeze dried fat body; *p*NP = *p*-nitrophenol.
* Significantly lower (t = 0.05) than the corresponding control values.
** Significantly higher (t = 0.05) than the corresponding control values.

diford, 1985). The appearance of two smaller peaks at 102 and 126 h, which have not been previously reported in any lepidopteran, may have been found as in our study the samples were drawn at shorter intervals than those reported by earlier workers (Barnby & Klocke, 1990). The occurrence of two distinct peaks in Lepidoptera (Bollenbacher et al., 1975; Lafont et al., 1977; Dean et al., 1980) may be advantageous because it provides a mechanism for adjusting development to environmental condition.

In plumbagin-treated insects, the temporal delay in the appearance of the peaks is not evident and none of the four peaks noticed in the control were lost as feeding was not drastically affected, unlike with the azadirachtin-treated insects. There were five ecdysteroid titers peaks and the fifth peak (unlike four in the control) occurred during the extended larval stage. Consequently, the gut-purge typical of control insects at 84 h was not noticed in the plumbagin treated larvae. The overall profile presents a reduced ecdysteroid titer. The action of plumbagin on ecdysteroid production is linked to the formation of dark material in the prothoracic glands, and is specific to certain exopterygotes. However, this is not observed in holometabolous insects (Joshi & Sehnal, 1989). There is ample evidence for the delay in build up and inhibition of A-type neurosecretion release in *H. armigera* (Krishnayya & Rao, 1995) and *Oncopeltus fasciatus* (Gujar & Dorn, 1996). The suppression of ecdysteroid titer observed without a concurrent darkening of the prothoracic glands may be due to the direct influence of plumbagin on these glands, in addition to its effect on the neurosecretory activity of the insect brain.

It has been shown that azadirachtin treatment modifies ecdysteroid titer in the bug, *O. fasciatus* (Redfern et al., 1982), the migratory locust, *Locusta migratoria* (Sieber & Rembold, 1983), the tobacco hornworm, *Manduca sexta* (Schluter et al., 1985) and in *Tenebrio molitor* (Marco et al., 1990), mainly by delaying the occurrence of the hemolymph ecdysteroid peak. Azadirachtin also acts as an anti-ecdysteroid in the blow fly, *Calliphora vicina* (Kauser & Koolman, 1983).

Azadirachtin-treated *H. armigera* had four peaks of hemolymph ecdysteroid titer, two in the normal and two in the extended larval stage, suggesting a temporal delay in the appearance of the last two peaks. None of the peaks exceeded 300 $ng \cdot ml^{-1}$. As with plumbagin treatment, the overall profile presents a reduced ecdysteroid titer. A precise matching of the ecdysteroid peaks with pre-metamorphic changes starting from gut-purge to pupation is not possible since these events could not be clearly demarcated in the treated insects.

Azadirachtin has no direct effect either on the prothoracicotropic hormone or the prothoracic gland secretion (Koul et al., 1987). It has no cross reactivity with ecdysone (Sieber & Rembold, 1983). Reduced feeding in azadirachtin-treated insects leads to sub-optimal body weights prior to molt initiation and the failure of cocoon spinning. This is primarily responsible for the temporal delay in the ecdysteroid peaks. Therefore, the results suggest that azadirachtin treatment affects the pre-pupal surge of ecdysteroid. The formation of larval-pupal intermediates is possibly due to the absence of the required titer of ecdysteroid needed for normal pupation. Most of the azadirachtin-treated insects had lost their exuvial fluid instead of resorbing it, as the latter process is also triggered by declining ecdysteroid titers (Truman, 1981). Thus, it is likely that pupation in azadirachtin-treated larvae is inhibited by disturbance in ecdysteroid regulation shortly before pupal ecdysis. Sieber & Rembold (1983), Subrahmanyam et al. (1989) and Subrahmanyam & Rembold (1989) demonstrated that azadirachtin injection leads to the inhibition of neurosecretion (prothoracicotropic hormone) turnover in the brain-corpora cardiaca complex, causing the inhibition or delay of a number of physiological processes. Although both plumbagin and azadirachtin treatments significantly reduced ecdysteroid titers in the hemolymph, based on the available evidence, it can be stated that they act at different loci.

The pre-metamorphic lysosomal activity in the fat body is characterised by changes in the activity of acid phosphoatase and β-galactosidase (Verkuil, 1978, 1980). Two peaks in acid phosphatase activity, one at the wandering stage and the other immediately after the larval-pupal molt, have been observed. These two peaks of enzyme activity have closer links with the two critical ecdysteroid peaks observed in this investigation. The first critical peak of ecdysteroid activity commenced at 60 h and extended up to 102 h during which time there was a remarkable increase in acid phosphatase activity greater than in the preceding and succeeding stages, until pupation. This suggests that ecdysteroids triggered an increase in acid phosphatase activity during epidermal reprogramming from larval to pupal cuticle.

The proliferation of lysosomes in response to the increase in 20-hydroxyecdysone may be due to the induction of hydrolase synthesis (Sridhara, 1981). Similar ecdysteroid mediated increases in the activity of lysosomal enzymes in insect fat body have been reported in *Stomoxys calcitrans* (Deloach & Mayer, 1979), *Corcyra cephalonica* (Ashok & Ray, 1988), *M. sexta* (Caglayan, 1990) and *Spodoptera litura* (Prasada Rao, 1990). Their studies demonstrated enhanced acid phosphatase activity due to the injection of 20-hydroxyecdysone into ligated insects. After the second critical ecdysteroid peak and the completion of the larval-pupal molt, there was an appreciable increase in acid phosphatase activity that may be involved in the histogenesis of adult structures.

β-galactosidase activity increased appreciably in the cocoon formation stage, coinciding with the first critical peak in ecdysteroid activity. However, the major and broad peak in β-galactosidase activity was seen starting from the early pre-pupal stage, and remained high in the freshly molted pupae. The significant rise in the early and late pre-pupal stages corresponds to a second critical ecdysteroid peak observed at 144 h which had a pronounced influence on this enzyme. This corroborates the findings of Prasada Rao et al. (1984) and Sharan et al. (1995) in *Spodoptera litura* and *Antheraea mylitta* respectively, who demonstrated the enhancement of β-galactosidase activity in ligated insects following 20-hydroxyecdysone injection. The first ecdysteroid peak had a relatively lesser influence on β-galactosidase activity than the second peak. Both enzyme activities were very low during the active feeding stage of the larvae, concomitant with reduced ecdysteroid titers.

A sharp increase in the protein content of the fat body was observed in the late pre-pupal stage, mainly due to sequestration of hemolymph protein by the fat body prior to larval-pupal transformation. This sequestration of haemolymph protein is closely associated with a second critical peak of ecdysteroid activity. Although the protein content was significantly higher at 120 h in both treatments, the activity level of the two enzymes did not increase. These changes in specific activity may be due to (a) an increase in enzyme activity, (b) a decrease in protein content, (c) a shift in enzyme distribution or (d) the de novo synthesis of enzymes. The results suggest that the activity of the two lysosomal enzymes is regulated by dynamic changes in ecdysteroid titer and that the two insect growth regulators have a profound influence on ecdysteroid mediated enzymatic physiological changes.

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