

**Activity of phosphatases and esterases in the aphid, *Acyrtosiphon pisum*
(Hemiptera: Sternorrhyncha: Aphididae), and in the gut wall
of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae and pupae**

VÁCLAV NĚMEC¹ and JAN ŽENKA²

¹Institute of Entomology, Czech Academy of Sciences, Branišovská 31,
370 05 České Budějovice, Czech Republic

²Bezdvorská 15, 370 11 České Budějovice, Czech Republic

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Abstract. The activity and isozyme patterns of esterases and phosphatases which hydrolyse ecdysteroid conjugates were studied in whole body extracts of *Acyrtosiphon pisum* (Harris) and in midgut wall extracts of *Galleria mellonella* (L.). The activity of esterases was almost ten times higher than that of phosphatases (expressed in μM liberated para-nitrophenol/min/mg protein) and seemed to decline with insect age. Cholinesterase sensitive to eserine comprised 14% of total esterase activity in the nymphs of *A. pisum*, while in other samples it was negligible. Activity of acid phosphatases in the aphid samples was twice as high as that of alkaline phosphatases, while a reverse 3 : 1 ratio was found in the wax moth. The alkaline phosphatases revealed higher sensitivity to fluoride than the acid phosphatases. Esterases were more variable in their isozyme patterns during development than the phosphatases. A possible mechanism of ecdysteroid mobilization from their conjugates and the role of hydrolyzing enzymes in these processes is discussed.

INTRODUCTION

Certain insect herbivores consume food containing ecdysteroids, which occur in plants in the form of polar hydroxyderivatives and phosphates (Grebenok et al., 1994). Similar conjugates produced in insect ovaries are deposited in the oocytes during vitellogenesis (Dinan & Rees, 1981, for survey see also Hoffmann & Lagueux, 1985). Scalia et al. (1987) and Slinger & Isaac (1988a, b) showed that the activity of enzymes hydrolyzing ecdysteroid conjugates fluctuates in parallel with conjugate utilisation and the titre of free ecdysteroids during embryogenesis.

Acetylation, phosphorylation and epimerization are the ways of ecdysteroid inactivation (Rees & Isaac, 1984, 1985; Rees, 1995). Locust nymphs were shown to possess metabolic pathways for ecdysteroid mobilization and inactivation similar to those in the embryos (Gibson et al., 1984; Modde et al., 1984), and it is probable that such a system occurs also in other developmental stages and in other insect species (Lafont & Connat, 1989).

Whilst studying the activity of phosphatases and esterases we tested:

- a) if the studied species have a capacity to mobilize ecdysteroids from the most frequently occurring ecdysteroid conjugates, i.e. phosphates and acyl esters and
- b) whether the activity of these hydrolyzing enzymes changes with the body titre of ecdysteroids.

MATERIAL AND METHODS

Sample preparation

Nymphs and adults of *Acyrtosiphon pisum* (Harris) were taken from a laboratory stock culture. They were reared under 16 h photoperiod at 19–21°C and 70–80% of relative humidity. About 70–80 nymphs (mixed stages) and 30 adults were taken for each assay. Collected aphids were kept at –20°C until use. Each sample was homogenized in 3 ml bi-distilled water chilled with ice. The homogenates were centrifuged and the supernatants were used for the enzymatic assays.

The wax moth *Galleria mellonella* (L.) was reared according to Sehnal (1966). Each sample represented the homogenate of one larval or pupal midgut. The guts of final instar larvae 3–4 days old (period of low ecdysteroid titre) and 6 days old (the time of ecdysteroid rise) and pupae 1 day old (low titre) and 96 h old (ecdysteroid peak, Sehnal et al., 1986) were cut open, and washed in ice-cold insect isotonic solution. Clean midgut wall was stored at –20°C and processed similarly as the aphid samples.

Protein determination

Protein content in homogenates was determined according to the method described in Goa (1953) after partial hydrolysis of the samples with 1.0 M NaOH overnight at room temperature. Bovine serum albumin was used as protein standard. Optical density was measured at 330 nm.

Enzyme assays

The activity of phosphatases was determined by the method of Linhardt & Walter (1965) modified for microanalysis by Němec & Socha (1988). Para-nitrophenylphosphate sodium salt (p-NPP) employed as substrate was dissolved in 0.05 M citrate buffer (for acid phosphatase) or 0.05 M glycine buffer (alkaline phosphatase). Sample aliquotes of 25 µl were taken for each assay. Incubation at 25°C was stopped after 60 min by transferring samples to a water bath (90°C) for 5 min. Optical density was read at 405 nm at the room temperature. The average of three triple sets of assays with various substrate concentrations were taken for the calculation of enzymatic reaction kinetic parameters. Activity was expressed according to Hanes (1932) or Lineweaver & Burk (1934) in µM of liberated para-nitrophenol per 1 mg of sample protein during 1 min. Data processing was performed using LABCHEM PC program (Ženka, 1994). Fluoride in concentration 20 mM NaF was used for estimating the participation of lysosomal phosphatases in the total activity (Aidels et al., 1971).

Esterase activity was measured using para-nitrophenylacetate (p-NPA) as a substrate (Huggins & Lapidus, 1947; modified by Němec, 1972). The samples were incubated for 30 min at 25°C, the reaction terminated by heating to 90°C for 5 min, and the optical density of cooled samples read at 405 nm. Calculation of the kinetic parameters of esterase was performed in the same way as used for the phosphatases. Eserin in final concentration 1×10^{-5} M was used to recognize the participation of cholinesterase in the total esterase activity.

Electrophoretic analysis of isozymes

Separation of phosphatase and esterase isozymes was performed by electrophoresis on 7% polyacrylamide gel (PAGE) according to Williams & Reisfeld (1964). Both acid and alkaline phosphatases were visualized according to Loxdale (1983), using 1,2-(alpha and beta) naphthylphosphate in 0.2 M veronal-acetate buffer (pH = 6.0 for acid, pH = 8.0 for alkaline phosphatases) and Fast Blue BB as a coupler. Esterases were detected with alpha and beta naphthylacetates dissolved in 0.2 M phosphate buffer (pH = 7.3 and incubated in darkness). Alpha and beta esterases (and phosphatases) were revealed as black and red stripes, respectively. Than the gels were differentiated using the mixture: methanol + water + acetic acid (5 : 5 : 1).

RESULTS

Comparing the acid and alkaline phosphatase activity in the nymph and adult pea aphid *Acyrtosiphon pisum*, it is evident from the V_{max} values that adults have more active enzymes (Tabs 1, 2, N, I). The nymphs have more lysosomal (fluoride-sensitive) phosphatases than adults. Esterase activity in the nymphs is nearly twice as high as it is in adults (see Tab. 3.).

TABLE 1. Acid phosphatase activity in nymphs and imagos of *A. pisum*, and in the gut wall of *G. mellonella* larvae and pupae.*

Sample	pH optimum	K _M	V _{MAX}	% inhibition
<i>A. pisum</i>				
Nymphs	5.0	132.0	5.55	14.3
Imagos	5.0	151.0	8.76	2.0
<i>G. mellonella</i>				
Larvae day 6	5.5	723.0	2.45	19.6
Larvae day 3	5.5	120.2	11.97	3.4
Pupae day 4	6.1	520.0	1.54	28.6
Pupae day 1	6.1	77.1	4.01	6.4

*K_M is given in μM of p-nitrophenol; V_{MAX} in μM liberated p-nitrophenol/1 min/1 mg sample protein; % inhibition expresses activity decrease in samples containing 20 mM NaF.

The affinity of phosphatases from the gut wall of wax moth larvae to the substrate is about 20% lower than those from the gut wall of pupae whereas the fluoride-sensitive phosphatases are higher in the larvae (Tabs 1, 2).

Comparing the activity of acid phosphatase in the gut wall of *Galleria* larvae and pupae at the ebb (L-3, P-1) and at the time of ecdysteroid titer rise (L-6, P-4) revealed higher activity in the former (Tab. 1, Fig. 2), but the fluoride-sensitive fraction was higher at the time of ecdysteroid increase. Alkaline phosphatase showed higher activity at the time of increased ecdysteroid titer, particularly its fluoride-sensitive fraction (Tab. 2, Fig. 3).

The activity of esterases in the gut wall of pupae represented only 20% of that found in the larvae, but the affinity for the substrate was higher in the pupae (Tab. 3 L, P). Cholinesterase represented only a small part of the total esterase activity.

The difference in esterase activity between day-2 larvae and day-1 pupae, and the activity at the time of ecdysteroid peak (L-6, P-4) was greater than established for alkaline phosphatase: more than 8 times higher in the gut wall of larvae and about 3.5 times greater in the gut wall of pupae (Tab. 3, Fig. 4).

The esterases in the samples from aphids were very active, particularly in the nymphs (about twice greater than in adults, see Tab. 3). Cholinesterase represented about 14% of the total activity in the nymphs, but only 3% in the adults. The pH optimum values were close to the actual pH of the studied samples (compare Tab. 1 with Tab. 3).

TABLE 2. Alkaline phosphatase activity in nymphs and imagos of *A. pisum*, and in the gut wall of *G. mellonella* larvae and pupae.

Sample	pH optimum	K _M	V _{MAX}	% inhibition
<i>A. pisum</i>				
Nymphs	8.2	124.0	2.74	45.5
Imagos	8.2	107.0	2.98	18.6
<i>G. mellonella</i>				
Larvae day 6	9.3	740.0	8.20	28.0
Larvae day 3	9.3	121.8	2.02	18.5
Pupae day 4	8.2	579.0	7.35	32.2
Pupae day 1	8.2	202.5	4.37	26.6

For explanations see Table 1.

TABLE 3. Esterase activity in nymphs and imagos of *A. pisum*, and in the gut wall of *G. mellonella* larvae and pupae.

Sample	pH actual	pH optimum	K _M	V _{MAX}	% inhibition
<i>A. pisum</i>					
Nymphs	6.81	6.6	784.0	299.1	13.7
Imagos	6.88	7.0	468.0	162.8	2.6
<i>G. mellonella</i>					
Larvae day 6	7.53	7.5	292.3	313.2	2.0
Larvae day 3	7.55	7.5	25.9	38.0	—
Pupae day 4	7.50	7.9	174.6	56.3	5.2
Pupae day 1	7.52	7.9	37.4	15.9	—

The column % inhibition indicates activity decrease in samples containing 10 μ M eserlin. For other explanations see Table 1.

The younger stages of examined insects (nymphs of aphids and larvae of the wax moth) showed higher enzymatic activity and more complex patterns of isozymes than the older stages (aphid imagos and wax moth pupae), as is evident from Fig. 1a, b.

The pattern of isoesterases in aphid nymphs was similar to that in adults, only alpha esterases close to start were absent in adults. A greater difference was found between the zymograms from wax moth gut wall: the stripes in the larvae were more intensive and condensed, while in the pupal samples they appeared indistinct and rather diffuse (Fig. 1).

DISCUSSION

We found that activity of alkaline phosphatases and esterases increases in *Galleria mellonella* midgut wall when the titer of ecdysteroids rises on day 6 of the last instar larvae

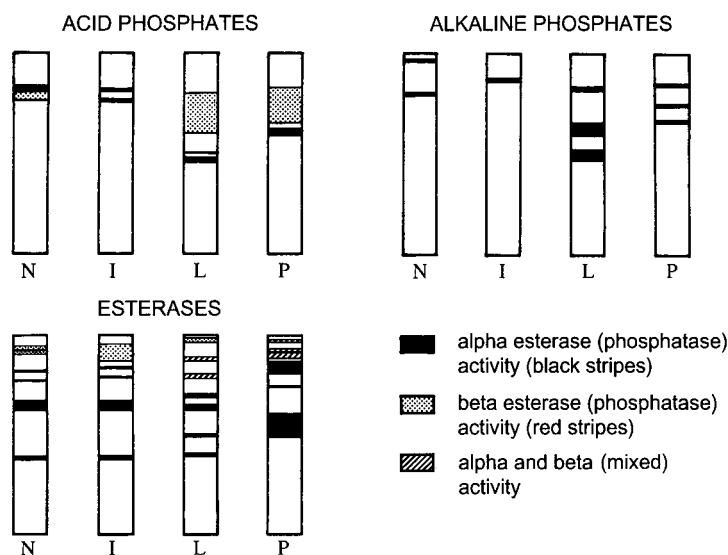


Fig. 1. Isozyme patterns of acid phosphatases (a), alkaline phosphatases (b) and esterases (c), prepared from *A. pisum* nymphs (N), adults (I) and the gut wall of *G. mellonella* larvae (L) and pupae (P).

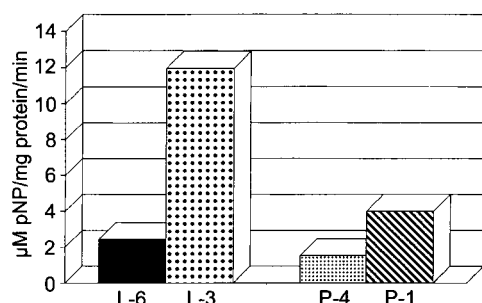


Fig. 2. Acid phosphatase activity (expressed as V max in μM of para-nitrophenol [p-NP] per mg of sample protein per min) in the gut wall of *G. mellonella*: L-6 – last instar larvae 6 days old (at the time of ecdysteroid rise). L-3 – last instar larvae 3–4 days old (low ecdysteroid titre). P-4 – pupae 96 h old (at the time of ecdysteroid peak). P-1 – the pupae at 24–48 h old (low ecdysteroid titre).

and in 96 h old pupae (see Tab. 2, 3, Figs 3, 4). It is in good agreement with the data of Sehnal & Žitňan (1990) who found that ecdysteroids are released from the wax moth gut wall at the rate 15 ng/h. A possible way of ecdysteroid mobilization is the hydrolysis of ecdysteroid conjugates as reported by Isaac et al. (1983) and, Sall et al. (1983) for locust embryos, by Thomson et al. (1988) for the embryos of the hornworm *Manduca sexta* and by Moribayashi & Ohtaki (1980) for the pupae of the flesh fly *Sarcophaga peregrina*.

Highest activity of acid phosphatases was found in wax moth midgut at the time of ecdysteroid rise (Tab. 1, Fig. 2). This is consistent with the reports that acid phosphatases reach their maximum activity around moulting (Lambremont, 1960), particularly when metamorphosis occurs (Lockshin & Williams, 1965).

The metabolic activity calculated per mg of sample protein is higher in small animals than in larger ones, and young developmental stages are usually more active than the old ones (Wigglesworth, 1965). In this study this was confirmed with alkaline phosphatase and with esterase in the gut wall of wax moth larvae and pupae (Tab. 2, 3).

The isozyme patterns of phosphatases and even esterases in the nymphs and adults of the pea aphid, *Acyrtosiphon pisum* were similar, differing only in several bands: nymphs have one beta-acid phosphatase, while imagos possess alpha-phosphatase (Fig. 1a). In respect to alkaline phosphatases, nymphs have an additional band (nearby start) absent in adults (Fig. 1b).

The esterase isozyme patterns in the aphid nymphs and imagos are very similar (Fig. 1c). This may be due to their long-time maintenance as parthenogenetic generations in our stock culture.

Samples from the gut wall of wax moth larvae exhibited more complex isozyme patterns than those from the pupae (Fig. 1a, b, c, L, P). Acid phosphatases in the larvae had three bands, and in the pupae only two.

In alkaline phosphatases, both larvae and pupae showed three bands, but they differed in their mobility. The patterns of esterases in the wax moth larvae and

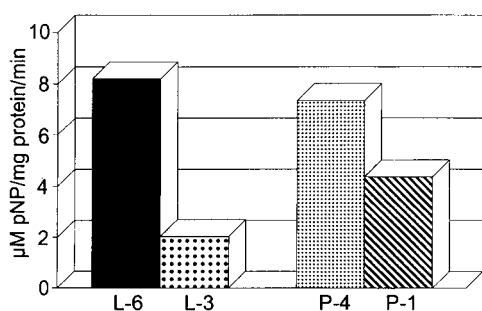


Fig. 3. Alkaline phosphatase activity (expressed as V max in μM of liberated p-NP/mg sample protein/min) in the gut wall of *G. mellonella* larvae and pupae. Explanations as in Fig. 2.

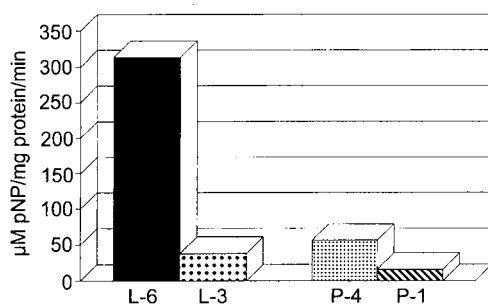


Fig. 4. Esterase activity (expressed as V_{\max} in μM of liberated p-NP/mg sample protein/min) in the gut wall of *G. mellonella* larvae and pupae. Explanations as in Fig. 2.

pupae did not differ in the number of bands but in their width and mobility: the two bands in pupae (esterase 5 and 7) were wide and rather diffuse, while in larvae they were sharp and narrow (Fig. 1c, L, P). Aphid nymphs and

adults also differed in the appearance of esterase bands (Tab. 3). A similar pattern was also found in alkaline phosphatase even though the differences were less clearly expressed (Tab. 2).

Pathways of ecdysone metabolism are not universal in all insects. They vary with species, developmental age and even with tissues of a given species (Lafont & Connat, 1989). The conjugation of ecdysteroids occurs mostly in the gut and Malpighian tubules, and to a lesser extent in the fat body (Koolman & Karlson, 1985). Conjugation of ecdysone with phosphate and fatty acids (esterification on C-3 and C-22) is most frequent among the other metabolic reactions (Connat & Diehl, 1986; Lafont & Connat, 1989; Grau & Lafont, 1994).

This study brings some indirect evidence that there is a physiological mechanism of ecdysone activation, and deactivation, via hydrolysis of ecdysone conjugates and/or esterification of free ecdysteroids as mentioned by Rees (1995). However, the metabolism of ecdysteroids is still obscure and requires further research.

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