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Soluble proteins and lipoproteins in Pieris brassicae (L.) (Lepidoptera, Pieridae): developmental changes and distributional differences

SEPPO TURUNEN

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Metamorphic changes in soluble proteins and lipoproteins of haemolymph, distribution of soluble proteins among and within other tissues (fat body and midgut), and the effect of dietary deficiency of lipids on haemolymph proteins and lipoproteins were examined with acrylamide gel electrophoresis in *Pieris brassicae*. The last larval ecdysis, pupation and adult emergence were associated with notable changes in the pattern of haemolymph proteins and lipoproteins. The patterns of soluble proteins in the haemolymph, fat body and midgut of fifth instar larvae were markedly different. Haemolymph lipoproteins resolved into four bands late in the fourth instar but into nine or ten bands late in the fifth instar and in pupae. Slowly migrating lipoproteins were prominent in newly oviposited eggs and in fifth instar larvae, but were almost absent from ecdysing fourth-fifth instar larvae and 2-day pupae. In fifth instar larvae reared from eggs on a diet deficient in essential fatty acids the haemolymph proteins had lipid contents greatly below normal.

S. Turunen, Division of Physiology, Department of Zoology, University of Helsinki, P. Rautatiekatu 13, SF-00100 Helsinki 10, Finland.

1. Introduction

Larvae of holometabolous insects accumulate nutrient reserves in the fat body, mainly in the form of triglycerides, proteins, and glycogen. The changes occurring in insect haemolymph proteins during development (CHEN & LEVENBOOK 1966, WHITMORE & GILBERT 1974) can be related to the synthesis and release of proteins from the fat body and to selective sequestration of certain haemolymph proteins by the fat body (WYATT 1975). Among their functions haemolymph proteins serve as carriers for neutral lipids (e.g. CHINO *et al.* 1969), steroid hormones (CHINO & GILBERT 1971), and, for example, the insect juvenile hormones (WHITMORE & GILBERT 1972, KRAMER *et al.* 1974, GILBERT & CHINO 1975, KRAMER & CHILDS 1977).

In the present study soluble proteins and lipoproteins of haemolymph were examined in males of the butterfly, *Pieris brassicae* (L.). Certain haemolymph proteins were found to vary markedly in lipid content during development. Electrophoretic protein patterns of the fat body and midgut were compared with those of the haemolymph. Dietary deficiency of lipids was manifested in the lipid content of haemolymph proteins.

2. Material and methods

Pieris brassicae were from our laboratory-reared stock (16L : 8D, 23°, 65 % R. H.) maintained on a recently modified artificial diet (TURUNEN 1978). Two further test diets were prepared (both containing 0.3 % cholesterol): one "lipid-free", the other containing 0.1 % (v/w) linseed oil ("low lipid") (TURUNEN 1974).

Haemolymph samples were obtained from larvae into graduated micropipettes at the following stages of development: 9-day (full-grown) fourth instars, pharate fifth instars, ecdysing fourth-fifth instars, 48-h post-ecdysis fifth instars, 96-h post-ecdysis fifth instars (pharate pupae), and from 2-day pupae and 3-day post-emergence adults. The samples were diluted to 1 : 20 with cold 40 % sucrose solution and used for electrophoresis. Parietal (dorsal, pigmented) and perivisceral (ventral, non-pigmented) fat bodies and midguts of late wandering fifth instar larvae were dissected under cold 0.9 % NaCl solution, homogenized in the saline solution, and centrifuged at 10 000 g for 30 min. Batches of about 50 eggs were homogenized one day after oviposition and treated as above. Haemolymph

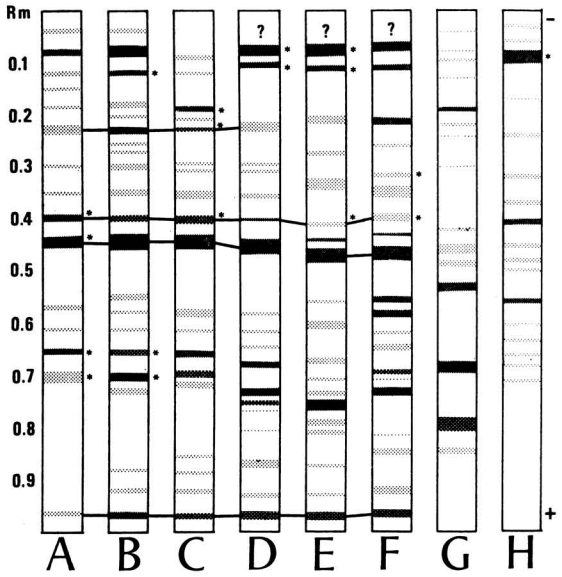


Fig. 1. Soluble proteins of haemolymph (A—G) and of eggs (H) in *P. brassicae*. A: 9-day 4th instar male larva, B: pharate 5th instar male larva, C: male larva at the time of the 4th-5th larval ecdysis, D: 48-h post-ecdysis 5th instar male larva, E: 96-h post-ecdysis male prepupa, F: 2-day male pupa, G: 3-day post-emergence male adult, H: 1-day oviposited egg.

samples (in 50 μl) spotted to each tube were 4 μl for soluble proteins and 5 μl for lipoproteins. The techniques followed in disc electrophoresis on acrylamide gel have been described (TURUNEN & CHIPPENDALE 1977). Bands of proteins and lipoproteins were detected with Coomassie blue and Sudan black, respectively (TURUNEN 1977).

3. Results

Fig. 1 shows the electropherograms of the major soluble proteins of haemolymph and of eggs. Throughout the larval and pupal stages studied the most densely staining soluble proteins occurred in three groups, one of which was slowly migrating (Rm 0.06 — 0.12), one intermediate (Rm 0.39 — 0.47), and one rapidly migrating (Rm 0.65 — 0.75). In adults, in contrast, the pattern was conspicuously different.

The soluble proteins of 9-day fourth instar larvae resolved into 15 bands (Fig. 1A), and the pattern was similar in pharate fifth instars (Fig. 1B). The concentrations of the slowly migrating proteins were distinctly decreased at the time of the last larval ecdysis (Fig. 1C). During the fifth instar electropherograms showed about 20 protein bands, with an increase in the number of rapidly migrating proteins (Fig. 1D, E). Pupation seemed to be associated with several changes, especially in the more rapidly migrating proteins (Fig. 1F). Possibly some rapidly migrating proteins (Rm 0.75 — 0.80) found in pharate pupae were not present in 2-day pupae. The patterns of soluble proteins of haemolymph also showed that striking differences were present after emergence of the adults (Fig. 1G). The number of protein bands had decreased to about 15 and the major haemolymph proteins of the adult male were rapidly migrating (Rm 0.68 and 0.80). In adult males the slowly migrating proteins formed only a minor constituent of the haemolymph. The soluble proteins of newly oviposited eggs, shown for comparison (Fig. 1H), are seen to differ profoundly from those of the adult male haemolymph.

Some of the changes observed in soluble proteins of the haemolymph during metamorphosis may have been related to the transport of lipids in haemolymph. This suggestion is supported by the changes observed in haemolymph lipoproteins (Fig. 2). Comparison of the mobilities of the proteins and lipoproteins (Figs. 1 and 2) suggests that several of the proteins shown in Fig. 1 are lipoproteins. On the basis of these data the major lipid-carrying proteins have been tentatively identified with (*) in Fig. 1.

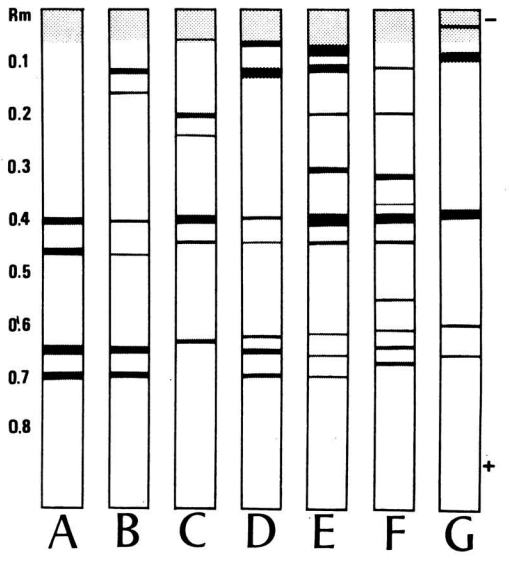
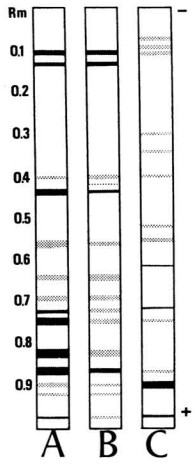


Fig. 2. Lipoproteins of haemolymph (A—F) and of eggs (G) in *P. brassicae*. Legend (A—F) as in Fig. 1.

In late fourth instar larvae no slowly migrating lipoproteins were detected in the haemolymph (Fig. 2A), although slowly migrating soluble proteins were present (Fig. 1A). At this stage, the four major haemolymph lipoproteins (Rm 0.40; 0.45; 0.65; 0.70) had the same mobilities as four of the soluble proteins. In pharate fifth instar larvae slowly migrating lipoproteins (Rm 0.12) were observed in addition to the four found in late fourth instar larvae (Fig. 2B). In ecdysing fourth-fifth instar larvae the pattern of lipoproteins was markedly different from that of fourth instar larvae (Fig. 2C). Lipoprotein Rm 0.12 was absent, which fits well with the decrease observed in the corresponding soluble protein at this stage (Fig. 1C). In ecdysing fourth-fifth instar larvae the appearance of two new lipoproteins (Rm 0.19 and 0.24) coincided with the presence of two distinct soluble proteins. In 48-h post-ecdysis fifth instars the lipoprotein at Rm 0.19 was no longer present, as shown by both protein and lipoprotein staining (Figs. 1D and 2D). This lipoprotein may have a specific function in connexion with larval ecdysis.

In 48-h and 96-h post-ecdysis fifth instar larvae the major lipoproteins migrate slowly (Fig. 2D, E). The lipid dye Sudan black stained nine bands in the haemolymph of 96-h post-ecdysis fifth instar larvae and 10 bands in the haemolymph of 2-day pupae (Fig. 2E, F). The slowly migrating proteins of 2-day pupae appeared to contain very little lipid, in marked contrast to those of fifth instar larvae. These proteins thus appear to be involved especially in transport of larval lipids. Both pharate pupae and 2-day pupae contained another major lipoprotein, which migrated more rapidly (Rm 0.40). This lipoprotein was found in the haemolymph of all developmental stages studied, but its lipid/protein ratio changed greatly during metamorphosis, being especially high in pharate pupae and in pupae. In contrast, lipoprotein Rm 0.45 appeared to have a rather low lipid/protein ratio throughout metamorphosis.

For comparison, electropherograms are presented to show the soluble proteins (Fig. 1H) and lipoproteins (Fig. 2G) of 1-day eggs (age from oviposition). A major slowly migrating lipoprotein (Rm 0.09) has a position identical with that of the major soluble protein. A more rapidly migrating egg lipoprotein (Rm 0.40) corresponds to the lipoprotein of comparable mobility found at all developmental stages studied.



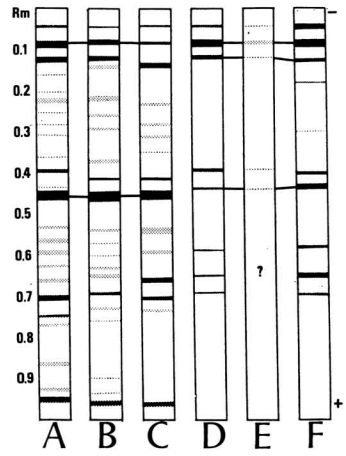


Fig. 3 (left). Tissue soluble proteins of late wandering male larvae of *P. brassicae*. A: dorsal parietal fat body, B: ventral perivisceral fat body, C: midgut.

Fig. 4 (right). Haemolymph soluble proteins (A—C) and lipoproteins (D—F) in 2-day post-ecdysis 5th instar male larvae of *P. brassicae* reared on lipid-deficient diets. A & D: control (standard diet), B & E: low-lipid diet (0.1 % linseed oil diet), C & F: larvae transferred from control diet to lipid-free diet on the 7th day of larval development.

Several differences in haemolymph proteins were seen between fifth instar larvae and pupae. For example, two rapidly migrating larval proteins (Rm 0.75 and 0.86) appeared to be absent from the pupae (Fig. 1D, E, F). Examination of late wandering fifth instar larvae showed that two proteins of comparable mobility (Rm 0.75 and 0.86) were also present in the dorsal parietal and ventral perivisceral fat bodies (Fig. 3A, B) and in the midgut (Fig. 3C). In these larvae the soluble proteins of the fat body were found to resolve into about 14 bands, the major proteins of the parietal tissue being rapidly migrating (Rm 0.44; 0.56; 0.75; 0.83; 0.86). Although they differed in appearance, the pigmented parietal and non-pigmented perivisceral tissues contained soluble proteins that were nearly identical in pattern.

In late wandering fifth instar larvae the soluble proteins of the midgut also resolved into 14 bands (Fig. 3C), but the electrophoretic pattern differed from that of the fat body. In the midgut, in contrast to the other tissues examined, the major soluble protein migrated

very rapidly (Rm 0.90). Almost nothing is known of the significance of the soluble proteins in the midgut tissue.

The concentration of haemolymph lipoproteins was distinctly influenced by the dietary lipid complement (Fig. 4). Only minor changes occurred in the soluble proteins of haemolymph when the larvae were reared on a diet containing a suboptimal level of the essential fatty acids (Fig. 4B), or when normal larvae were transferred to a lipid-free diet at the third instar and reared to the fifth instar (Fig. 4C). But these treatments led to distinct changes in haemolymph lipoproteins. Thus only small concentrations of lipoproteins were detected in larvae reared on the low-lipid diet (Fig. 4E). Rather unexpectedly, after the second treatment (in which larvae were transferred from the control diet to a lipid-free diet at the third instar) the lipid content of the haemolymph lipoproteins appeared to undergo a marked increase (Fig. 4F). On both these test diets larval survival was reduced, and after the second treatment pupation was almost completely suppressed (Table 1). Also, whereas the majority of larvae reared on the low-lipid diet eventually pupated, none pupated after being fed on a diet lacking essential fatty acids (Table 1, third column).

Table 1. Mean time (days) of development of *P. brassicae* larvae on lipid-deficient diets.

Stage	Diet[1]		
	Low lipid[2]	Lipid-free[3]	Control + Lipid-free[4]
1st ecdysis	4	4	3
2nd ecdysis	6	8	5
3rd ecdysis	9.5	12 (30 %)	8
4th ecdysis	15 (57 %)	22 (7 %)	11 (60 %)
Pupation	22 (50 %)	None	None[5]

From 70 to 90 1st instar larvae were placed on each diet

[1] All diets contained 0.3 % cholesterol

[2] Contained 0.1 % (v/w) linseed oil

[3] The only added lipid was cholesterol

[4] Larvae were transferred from the control diet to the lipid-free diet on the 7th day after hatching.

[5] In a few cases some diminutive pupae were obtained. Larvae reared on the control diet throughout the larval stage pupated in about 15 days. Values in parentheses indicate percentage of animals ecdysed or pupated by the time shown.

4. Discussion

These results showed that distinct changes occur in haemolymph proteins and lipoproteins of *Pieris brassicae* during development, especially at the time of the last larval ecdysis and in connexion with pupation. In addition, the storage of certain haemolymph proteins in the fat body of late fifth instar larvae, although not confirmed, could be inferred. The results further suggest that deficiency of dietary essential fatty acids is reflected in the lipid content of haemolymph proteins.

In an extensive study of haemolymph proteins and lipoproteins from seven species of Lepidoptera, WHITMORE & GILBERT (1974) found similarities in the protein patterns of closely related species. All the haemolymph lipoproteins in these species were found to be glycoproteins and some exhibited esterase activity. The species studied also showed changes in haemolymph protein pattern during development, in agreement with numerous other observations (CHEN & LEVENBOOK 1966, VINSON & LEWIS 1969, WYATT 1975).

The difficulty of establishing without immunological studies that identically located bands in the electropherograms are identical proteins interferes with the interpretation of the developmental changes. However, their staining properties and mobilities strongly suggest that several of the haemolymph proteins of *P. brassicae* are closely similar, if not identical, at the different stages of development (Fig. 1). None of the proteins seemed to be specific to larvae. The pattern in adult males was characteristically different from those seen in larvae or pupae, and one of the major adult proteins (Rm 0.53) could perhaps be classified as an adult protein. There have been several examples of proteins specific only for certain stages of development in insects (LAUFER 1960, FOX & MILLS 1969, KUNKEL & LAWLER 1974, KINNEAR & THOMPSON 1975). The significance of these proteins remains obscure, although they possibly act as nutrient reserves in some species (KUNKEL & LAWLER 1974). Recently a protein specific for the larval diapause of the corn borer, *Diatraea grandiosella*, has been described and observed to be under the control of juvenile hormone (BROWN *et al.*, in preparation).

CHIPPENDALE & KILBY (1969), working with *P. brassicae*, noted the selective uptake of two slowly migrating larval haemolymph pro-

teins by the fat body in late fifth instar larvae. Their data further suggest that these proteins are absent from the haemolymph of newly ecdysed pupae. The present results show that the major fat body proteins of late wandering fifth instar males were rapidly migrating and suggest that they originate from the haemolymph. Such selective storage of haemolymph proteins in the fat body has been reported in other species besides *P. brassicae* (TOBE & LOUGHTON 1969, MARTIN *et al.* 1971, WYATT 1975).

The purpose of the experiments with lipid-deficient diets was to examine the effects of fatty acid deprivation on internal tissue morphology and larval survival. When larvae were reared from eggs to fifth instars on a diet containing only 0.1 % (v/w) linseed oil, the internal tissues, e.g. the midgut, were fragile and the fat body was thin, with little visible storage material. Analysis of haemolymph lipoproteins suggested that in these larvae there was very little transport of fat. But when normal larvae were transferred to a lipid-free diet before the fourth instar and examined at the beginning of the fifth instar, the lipid content of the haemolymph proteins was found to be higher than in the controls. These data may indicate rapid transport of the diminished lipid stores to critical sites of tissue construction.

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