

**Purification and characterization of storage protein from the haemolymph
of *Mamestra brassicae* (Lepidoptera: Noctuidae)**

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Abstract. The storage protein of the cabbage moth (*M. brassicae* L.) was purified from larval haemolymph by anion exchange chromatography, followed by preparative electrophoresis and elution. Histochemical staining revealed that the storage protein is a glyco-lipoprotein. SDS-polyacrylamide gel electrophoresis suggested that this protein is composed of two dominant – M_r 81.9 and 74.6 kD – and five minor – M_r 64.1, 58.3, 50.1, 40.8 and 38.2 kD – subunits. Electrofocusing separation showed the presence of these subunits at pH 5.4–6.3. The conformation of the storage protein was equally stable at pH ranging from 4 to 9. The *M. brassicae* storage protein is immunologically related to *Lymantria dispar* arylphorin.

INTRODUCTION

It is well-known that storage proteins, especially prior to metamorphosis, belong to the quantitatively most conspicuous family of insect haemolymph proteins. In feeding larvae, storage proteins are synthesized primarily by the fat body and are secreted into the haemolymph (see Levenbook, 1985; Telfer & Kunkel, 1991). Their native molecular weight is around 500,000 (Wyatt & Pan, 1978). As was observed of various lepidopteran species, the storage proteins consist of only one type of subunit (*Hyalophora cecropia*, arylphorin, M_r 73 kD: Telfer et al., 1983; *Papilio polyxenes*, MSP-1, M_r 75 kD and MSP-2, M_r 74 kD: Ryan et al., 1986; *Pieris rapae*, SP-1, M_r 77 kD: Kim et al., 1989) or of more than one. Usually there were two types (*Manduca sexta*, arylphorin, M_r 79 and 74 kD: Riddiford & Hice, 1985; *P. rapae*, SP-2, M_r 69 and 80 kD: Kim et al., 1989; *Lymantria dispar*, arylphorin, M_r 73 and 80 kD: Karpells et al., 1990). Three abundant storage proteins have been detected in *Heliothis virescens*; these polypeptides have subunit molecular weights of 74, 76 and 82 kD (Leclerc & Miller, 1990).

There are several classes of storage proteins found in Lepidoptera. Arylphorins are distinguished by a high content of aromatic amino acids (Telfer et al., 1983). Female-specific proteins have a high frequency in females and are rich in methionine (Ryan et al., 1985). The latter proteins are in some cases referred to as larval serum proteins (Kunkel et al., 1990), while high-density lipoproteins are called lipophorins (Chino et al., 1981).

With regard to their function, development, and evolution, storage proteins are a complex family whose analysis promises to be very highly useful to the study of many basic problems in insect biology (Telfer & Kunkel, 1991). Molecular analyses of storage proteins and their genes are currently being made (see Leclerc & Miller, 1990; Corpuz et al., 1991). Changes in protein patterns following the infection of cabbage moth larvae with

nucleopolyhedrosis virus (Van Der Geest & Wassink, 1969), and both the quantitative and qualitative changes in haemolymph proteins during the two last larval instars and the early pupal stage (Slovák et al., 1992), have already been studied. The isolated storage protein has been observed by Sass (1988), but it has not been characterized in greater detail. The purpose of the present experiments was to purify and partially characterize the main protein of last instar larvae haemolymph in the cabbage moth.

MATERIALS AND METHODS

All chemicals used in this study were obtained from Sigma Chemical Co., unless otherwise specified.

Animals and haemolymph collection

Cabbage moth (*Mamestra brassicae* L.) (Lepidoptera: Noctuidae) specimens were obtained from permanent rearing in our laboratory. Larvae were reared at $24 \pm 1^\circ\text{C}$, $65 \pm 5\%$ RH and 8L:16D (light intensity about 1,600 lux). They were fed leaves of Brussels sprouts (*Brassica oleracea* var. *gemmifera* DC) from the time of hatching to the end of the 3rd instar, and thereafter on the semi-synthetic diet of Podmanická & Weismann (1975). From the 3rd instar, larvae were reared at a density of 3 individuals per Petri dish (d-10 cm). Insects were synchronized at the time of moulting to within ± 12 hr. This was done at each larval moult as well as at the pupal moult.

Haemolymph was collected on each day of development from newly moulted 5th instar larvae through to 3-day-old pupae (at least 6 individuals). Bleeding was performed by cutting the second abdominal proleg in larvae and prepupae, or by puncturing the thorax cuticle in pupae. A few crystals of phenylthiourea and 5 mM phenylmethylsulfonyl fluoride were added to the haemolymph collected in ice-cooled Eppendorf tubes. The haemolymph was then centrifuged at 7,800 g for 10 min. The supernatant was used immediately or stored at -35°C . The protein content was determined using the colorimetric method according to Bradford (1976), with BSA as the standard.

Purification of storage protein

The DEAE-cellulose (Whatmann) column (1.5 cm \times 18 cm) was equilibrated with 50 mM Tris-HCl and 1 mM EDTA pH 7.5. Samples (approx. 10 ml from 3 days old pupae) were dialyzed in 250 ml of the same buffer for approximately 5 hr, and then centrifuged again at 7,800 g. They were put into the column and eluted with a linear 200 ml gradient to a limit of 0.5 M NaCl in the same buffer, at a flow rate of 24 ml/hr. The eluant was monitored at 280 nm. Gradient 5–8% non-denaturing polyacrylamide gel electrophoresis (PAGE; see below) was used to test the column fractions of the protein peak for the presence of storage proteins.

The enriched storage protein (fraction 15) was then separated on a preparative (3mm thickness) gradient 4–10% non-denaturing PAGE, using the discontinuous buffer system (pH 6.8 and 8.8) of Laemmli (1970). Sodium dodecyl sulfate (SDS) and 2-Mercaptoethanol were, however, not used. The running buffer used was Tris-glycine (pH 8.3). Native proteins were electrophoresed for approximately 5 hr at 12 V/cm. After PAGE, the storage protein band was excised with a razor blade and eluted overnight into 50 mM of Tris-HCl buffer (pH 6.8) at 4°C .

The purity of the storage protein was tested by means of both a gradient 5–8% non-denaturing PAGE (gels were stained after electrophoresis with Coomassie brilliant blue R) and Western blotting (see below).

Production of antiserum

The polyclonal polyspecific antiserum was prepared using a rabbit (chinchilla breed, purchased from Breeding of laboratory animals, Lysolaje, Praha) injected with the haemolymph serum of 4-day-old 6th instar cabbage moth larvae. Five hundred μg (15 μl) of serum proteins were diluted to 2.5 ml by addition of PBS (pH 7.2). One hundred μg of serum proteins in 0.5 ml of this solution, emulsified with an equal volume of Freund's complete adjuvant, were injected subcutaneously as initial immunization. The rabbit was boosted twice (at three week intervals) with 100 μg of serum proteins in Freund's incomplete

adjuvant. Three weeks later an intravenous injection of 200 µg of serum proteins in PBS (without Freund's adjuvant) was administered. Blood from the heart was collected 2 weeks after the last injection and then allowed to clot one hour at 37°C and subsequently was clotted overnight at 4°C. After centrifugation for 10 min at 10,000 g, the resulting antiserum (supernatant to which 0.01% (w/v) of sodium azide was added) was stored at -37°C.

Western blotting

The electrophoretic transfer of proteins from non-denaturing gels to a nitrocellulose membrane (Schleicher & Schuell) was done at 4°C for 24 hr at 190 mA current (approx. 45 V), in a transblot cell with constant stirring of the transfer buffer (0.7% acetic acid). Following the transfer, the membrane was washed for 3 min in three changes of distilled water, and unoccupied binding sites of the nitrocellulose were blocked by incubation for 1 hr at 40°C in 100 ml of 5% BSA solution in TEN buffer (50 mM Tris-HCl pH 7.4, 5 mM EDTA, 150 mM NaCl and 0.05% (v/v) Tween 20, to which 100 µl of 10% (w/v) sodium azide were added). Next, the blot was rinsed for 3 min in three changes of distilled water, and air-dried or incubated in 100 ml of primary antibody buffer (1% BSA in TEN buffer, containing 100 µl of anti-haemolymph serum crude IgG) for 2 hr at room temperature. After being washed in TEN buffer (twice for 5 min and twice for 10 min), the blot was soaked in 100 ml of secondary antibody buffer (1% BSA in TEN buffer containing 50 µl of peroxidase conjugated swine anti-rabbit IgG (Institute of Sera and Vaccines, Praha) for 2 hr at room temperature, and washed 5 times in TEN buffer (10, 5, 5, 10 and 10 min). The peroxidase was made visible by staining with 0.04% (w/v) 3,3-Diaminobenzidine tetrahydrochloride (Fluka), 0.2% (v/v) hydrogen peroxide (33%), 0.03% CoCl₂ in 50 mM Tris-HCl pH 7.4. The reaction was stopped by rinsing in distilled water.

Characterization of storage protein

The purified storage protein was tested for the presence of lipo- and glyco-components (after the non-denaturing gradient 5–8% PAGE) by means of histochemical staining methods, as described by Slovák et al. (1992).

Molecular weight determination

The storage protein, denatured by heating (5 min) in a sample buffer (125 mM Tris-HCl pH 6.8, 20% v/v glycerol, 4% w/v SDS, 10% v/v 2-mercaptoethanol and a few crystals of Bromphenol blue), was subjected to electrophoresis at a constant current of 3 mA/cm in gels of 12% acrylamide, with an acrylamide/bisacrylamide ratio of 37.5:1, in the discontinuous buffer system of Laemmli (1970). Molecular weight standards (LMWS, Bio-Rad) phosphorylase B (97,400), bovine serum albumin (BSA, 66,200), ovalbumin (42,699), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400) were run in parallel lanes. A standard curve, constructed from their relative migration and known molecular weights, was used to estimate the molecular weight of storage protein subunits. After electrophoresis, the gels were incubated overnight in a fixative solution (40% v/v ethanol, 20% v/v acetic acid), and stained with silver according to Damerval et al. (1987). Slab gels were scanned by using a densitometric accessory to spectrophotometer PYE-UNICAM SP 8-100.

Determination of pI

Isoelectric focusing was performed according to a protocol provided by Hoefler (Giulian, 1990) on non-denaturing 5% polyacrylamide slab gels, using a 6% mixture of ampholytes pH 3–10 and pH 5–7 Ampholine (LKB) with a ratio of 1:1. After half an hour of prefocusing at a constant power of 0.7 W per cm gel width, the electrofocusing was run for 3 hr with a constant power of 0.5 W/cm. Equine myoglobin was used as an indicator. To monitor the pH gradient, 0.5 cm pieces were cut from a 1 cm-wide strip of electrofocusing gel. The slides were eluted overnight with 1 ml portions of distilled water, and pH was measured in the eluates. The gels were scanned using the Camag TLC Scanner II.

pH stability

After preparative PAGE and elution (as above), the samples were dialyzed against Davies's universal buffer (Čársky, 1981), with pH changed from 2 to 11 in increments of 1 pH unit for 20 hr at 25°C. The dissociation of storage protein was observed on 4–10% non-denaturing PAGE (as above).

Immunological studies

Double immunodiffusion was performed according to the method of Ouchterlony (1968), using 1% agarose in phosphate buffer pH 7.0, to which 0.02% (w/v) sodium azide was added. The antisera used in this study (rabbit anti-*Lymantria dispar* arylphorin, rabbit anti-*L. dispar* lipophorin and goat anti-*L. dispar* yolk protein) were obtained from the Zoology Department, University of Massachusetts at Amherst, USA. A serum of cabbage moth haemolymph, as well as purified storage protein of different dilutions (from 0 to 1:2,048), were used as antigens.

Immunoblotting was carried out as described above, but the electrophoretic transfer of proteins after SDS-denaturing 12% PAGE was performed in a 40 mM NaH₂PO₄ (pH 6.5) transfer buffer. Rabbit anti-*L. dispar* arylphorin and lipophorin were used in primary antibody buffers. Following their last washing in TEN buffer, the membranes were incubated for 1 min in 50 mM acetate buffer pH 5. The peroxidase was made visible by staining with 0.04% 3-Amino-9-Ethylcarbazole (dissolved in 5 ml of acetone), and diluted to 100 ml by the addition of 0.05 M acetate buffer pH 5 and 50 µl hydrogen peroxide.

RESULTS

Purification of the storage protein

The first step in the purification of the cabbage moth storage protein was fractionation of the clarified larval haemolymph using DEAE-Cellulose chromatography. As shown in Figure 1, one protein peak was eluted at approx. 0.06 M NaCl, and analytical PAGE

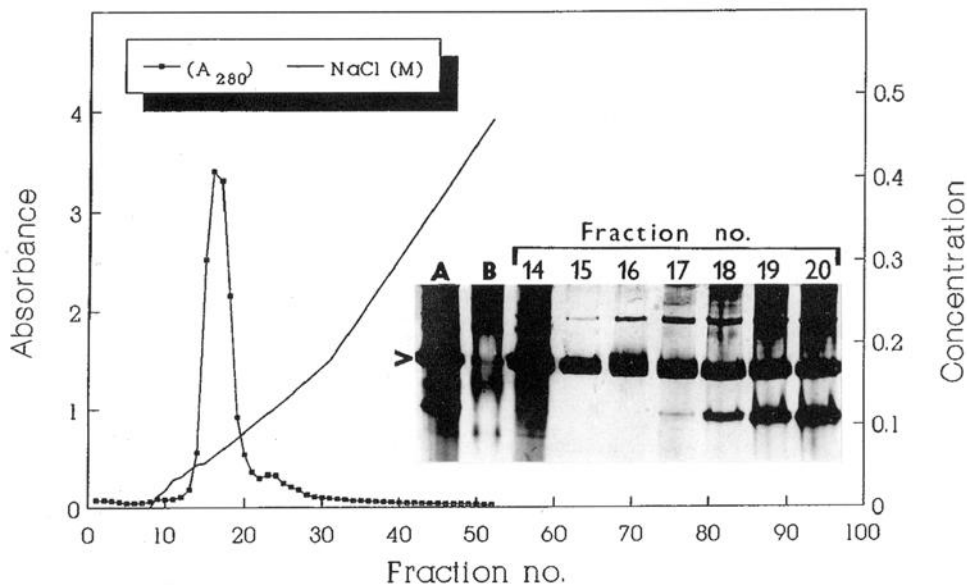


Fig. 1: Chromatographic profiles of cabbage moth haemolymph storage protein after ion-exchange chromatography on DEAE-Cellulose column. Insert shows 5–8% native PAGE profiles of peak fractions (10 µg) tested for the presence and purity of storage protein (>). A and B denote total haemolymph proteins and flowthrough, respectively. Gels were stained with silver.

showed that the storage protein was present in fractions from this peak. Because fraction 15 consisted of the fewest components, further work was concentrated on this fraction. Preparative PAGE, followed by elution, was used in the final step of the purification. The electrophoretogram of native proteins shown in Figure 2 depicts the progress of the purification at each step; lane 3 demonstrates the apparent purity of the storage protein preparation. This purity was confirmed by Western blotting with a polyspecific antiserum directed against proteins from the cabbage moth haemolymph serum (Fig. 2).

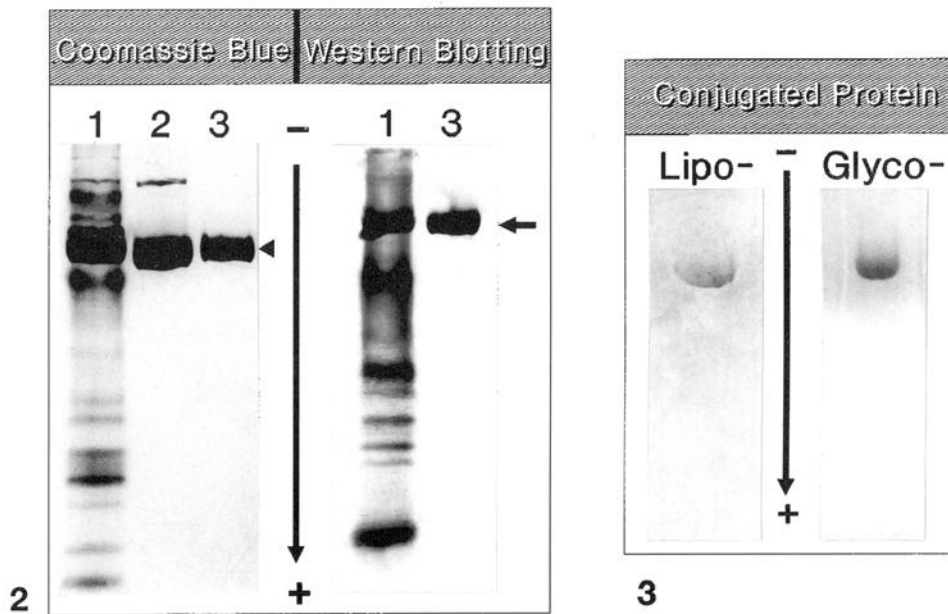


Fig. 2: Electrophoretic analysis of storage protein (arrows) following purification. Stained with Coomassie brilliant blue (after 5–8% native PAGE) or Western blotting with polyspecific antiserum (after 6–10% native PAGE). 1 – total haemolymph (150 μ g); 2 – DEAE-Cellulose fraction no. 15 (30 μ g); 3 – after electroelution (10 μ g). The immunoblotted gel was loaded with half as much of the sample volume as the Coomassie stained gel.

Fig. 3: Purified storage protein (100 μ g) after 5–8% native-PAGE (stained with Sudan Black B) and PAS procedure for identification of lipo- and glyco- components.

Characterization of the storage protein

Histochemical staining of the purified storage protein revealed that it is a conjugated protein containing both lipo- and glyco- components (Fig. 3). SDS-PAGE suggested that this protein is composed of two non-identical dominant subunits of MW 81.9 and 74.6 kD, as well as five minor subunits of MW 64.1, 58.3, 50.1, 40.8 and 38.2 kD (Fig. 4). An electrofocusing separation showed that these subunits have PI ranging among the pH 5.4–6.3 (Fig. 5).

When dialyzed against a series of buffers, the cabbage moth storage protein was dissociated into at least seven subunits (with relative mobility R_f 0.16, 0.23, 0.37, 0.49, 0.51, 0.54 and 0.60), depending on the pH of each buffer (Fig. 6). A change in pH from 4

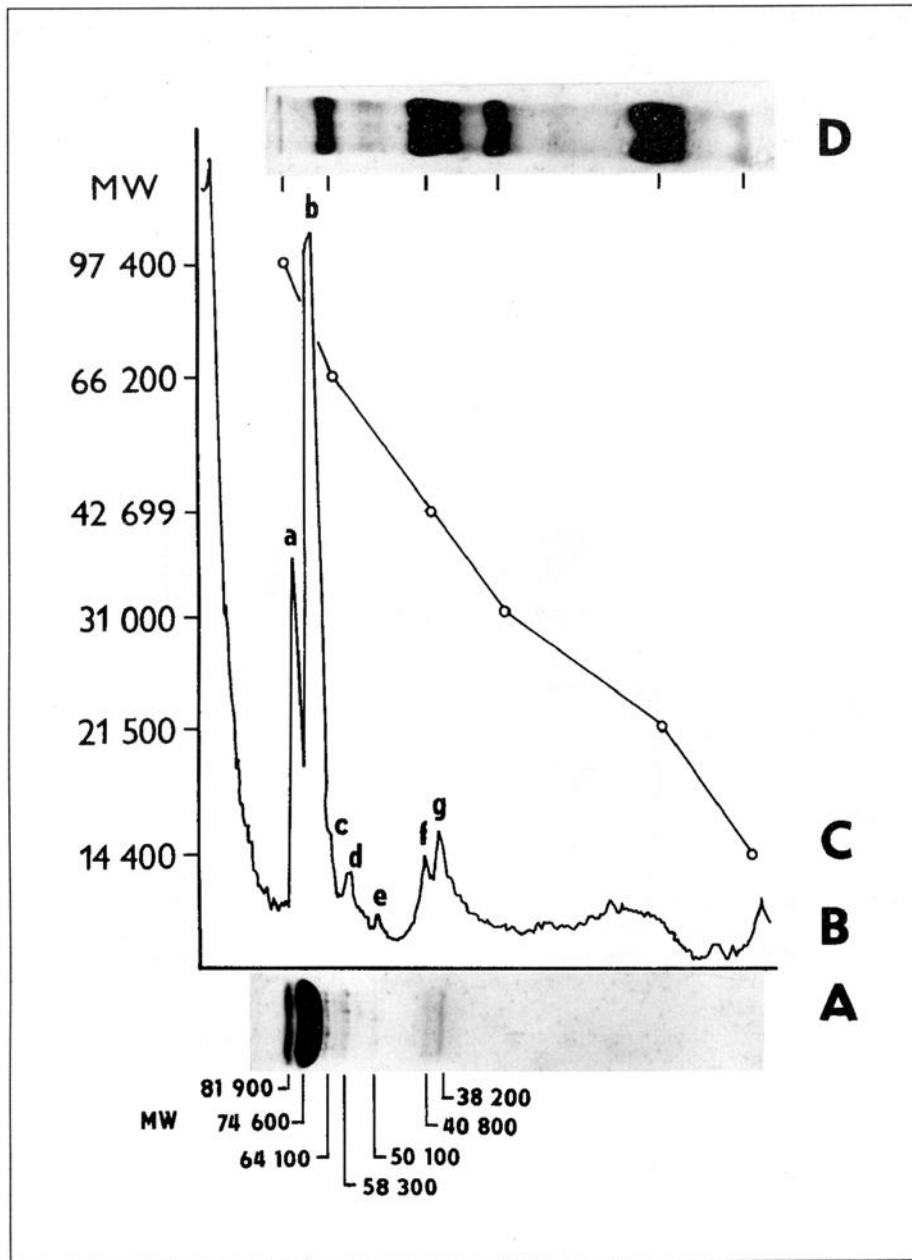


Fig. 4: Determination of molecular weights of cabbage moth storage protein subunits. A - storage protein subunits separated by 12% SDS-PAGE (5 μg of purified protein). B - printouts from scan of gel. Peaks a-g represent densitometric tracing of subunits. C - calibration curve for molecular weights marker proteins (MW in Daltons). D - SDS-PAGE of MW standards.

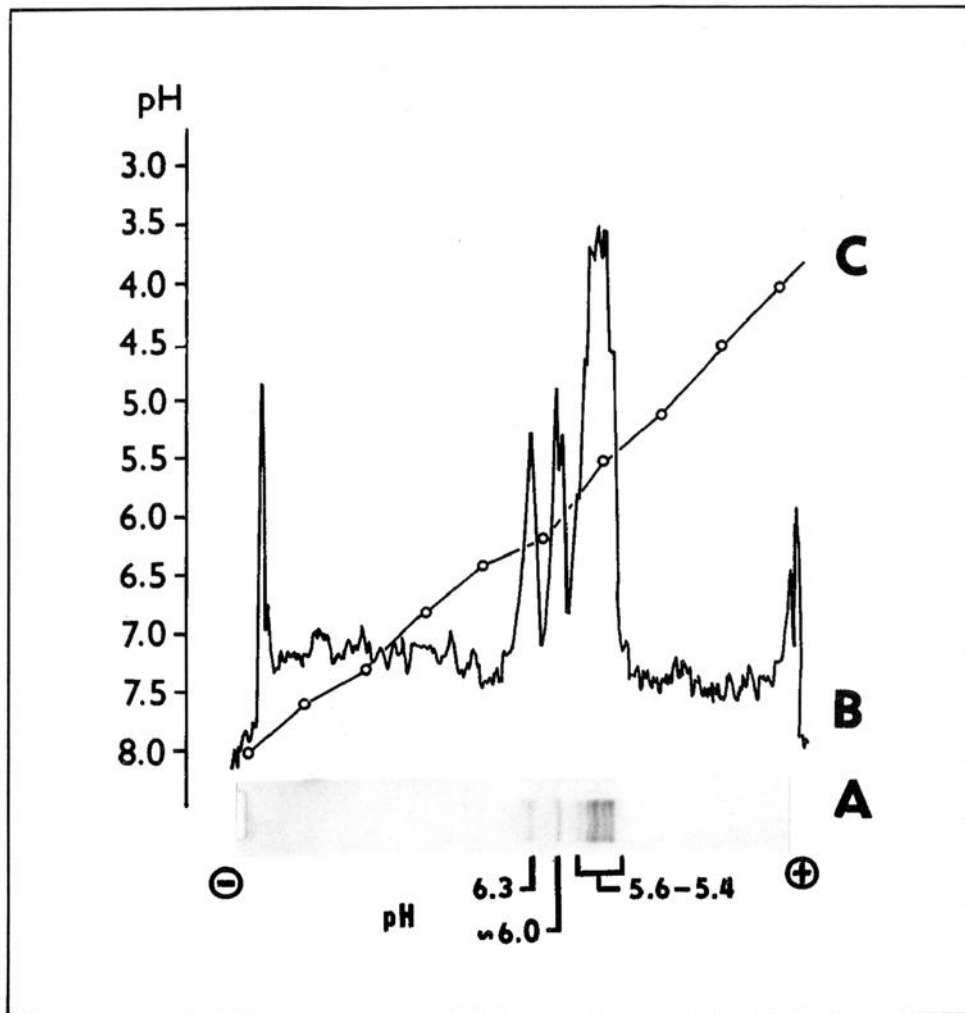
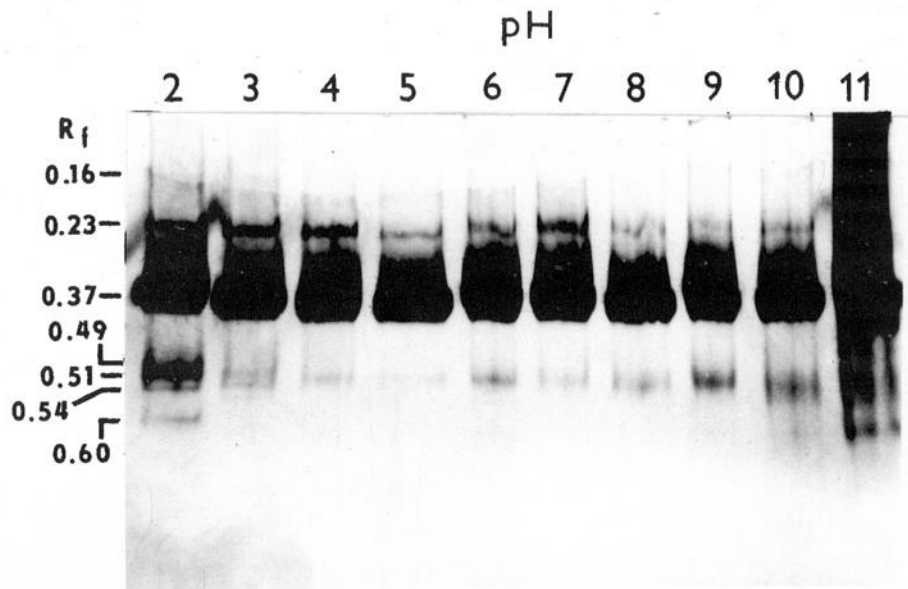
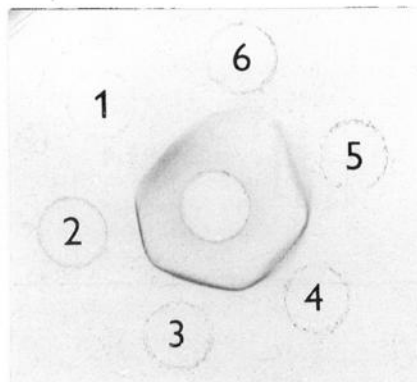


Fig. 5: Determination of isoelectric points (pI) of cabbage moth storage protein. A – purified storage protein (15 μ g) separated by isoelectric focusing. B – scan of an isoelectric focusing gel. Peaks represent densitometric tracing of subunits. C – calibration curve for pH gradient profile of the gel, using slides as described in Methods.

through to 9 was accompanied by progressive conformational changes (3–4 subunits) tending toward random structure. Major changes occurred at pH 2–3, pH 10 (5–7 subunits), and especially at pH 11, where the protein started to denature.



6



7



8

Figs 6-8. 6 - Dissociation of purified cabbage moth storage protein during dialysis against buffers of differing pH. Twenty μg of samples were tested after dialysis for the presence of subunits. The 4-10% non-denaturing PAGE gels were stained with silver. 7 - Ouchterlony double-diffusion analysis of the purified storage protein. The center well contained serum against *L. dispar* arylphorin (20 μl). Outer wells 1-6 were loaded with antigen in concentrations of 5.6, 2.8, 1.4, 0.7, 0.35 and 0.175 μg , respectively. 8 - Immunodetection of anti-*L. dispar* arylphorin specificity against native proteins of cabbage moth haemolymph. Fifty μg of haemolymph of 6th instar larvae on day 4 were electroblotted after 5-8% PAGE onto nitrocellulose, and immunostained for arylphorin.

Immunological studies

In immunodiffusion experiments, polyclonal antibodies directed against the lipophorin or yolk protein from *L. dispar* did not cross-react with either the cabbage moth or

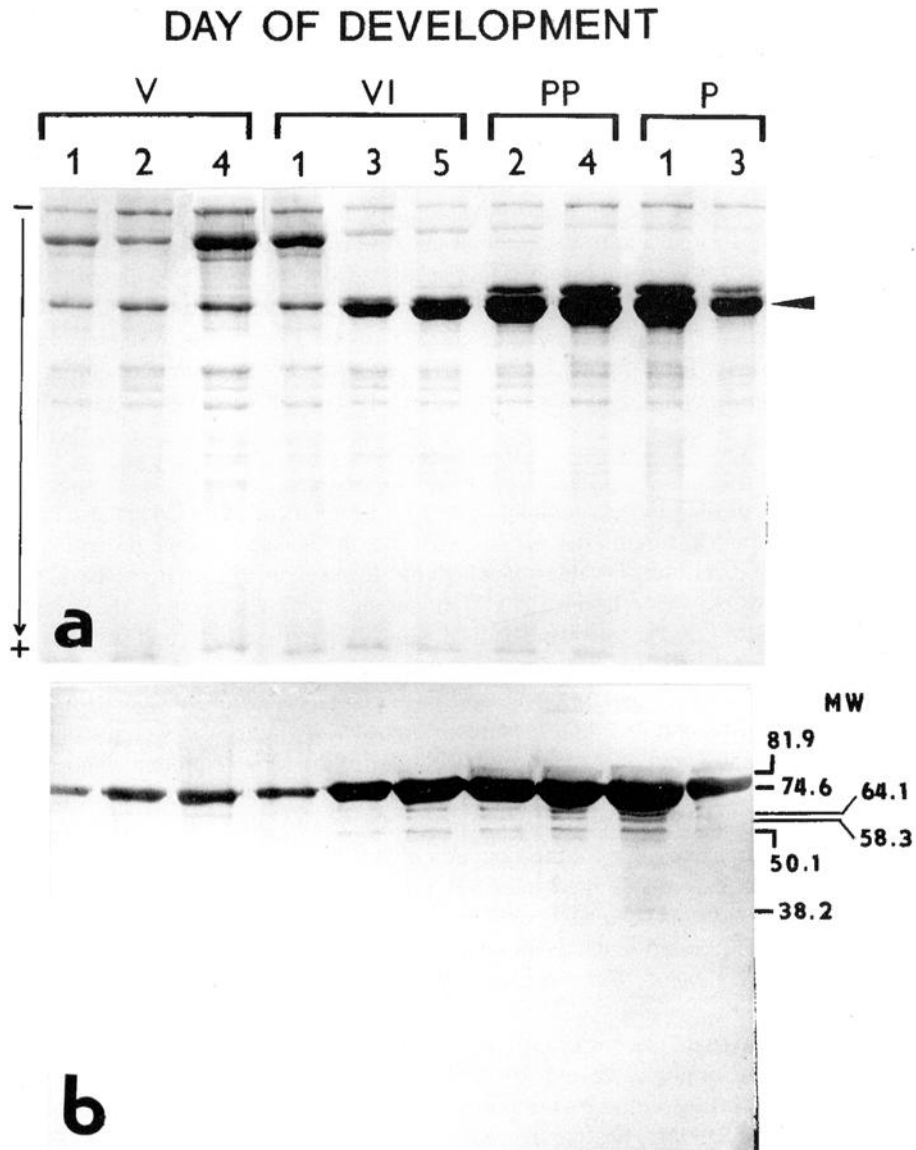


Fig. 9: Accumulation of cabbage moth storage protein in haemolymph during development from 5th instar larvae to pupae. After 12% SDS-PAGE of haemolymph serum proteins (10 µg/day), the gels were (a) stained with Coomassie brilliant blue or (b) electroblotted onto nitrocellulose and immunostained for arylphorin (antiserum used: anti-*L. dispar* arylphorin). V and VI indicate 5th and 6th instar larvae, respectively; PP – prepupae; P – pupae; arrow indicates the main subunit (74.6 kD) of storage protein.

haemolymph serum or purified storage protein (data not shown). However, these experiments revealed the immunological relationship of *L. dispar* arylphorin to the purified *M. brassicae* storage protein, yielding a single precipitation line (Fig. 7).

Immunoblotting experiments (after non-denaturing PAGE) confirmed that anti-*L. dispar* - arylphorin antiserum was specific to the storage protein and did not react with other cabbage moth haemolymph proteins (Fig. 8). At the same time, these experiments (after denaturing PAGE) showed that the cabbage moth storage protein is composed of subunits which are not equally synthesized during development (Fig. 9b). Along with the accumulation of the storage protein before moulting in the penultimate and last instar larvae (Fig. 9 a,b), there was an increase in the number of its subunits. One subunit (M_r 74.6 kD) was observed in the 5th and 6th instar larvae on day 1 (Fig. 9b). Two subunits (M_r 74.6 and the most probably 58.3 kD) were seen in the 5th instar larvae before moulting on day 4. The anti-*L. dispar* arylphorin revealed four subunits (M_r 74.6, 64.1, 58.3 and 50.1 kD) in the 6th instar larvae on day 5. The main subunit with M_r 81.9 kD became partially visible for the first time in prepupae on day 2. The greatest number of subunits were observable in pupae on day 1 (M_r 81.9, 74.6, 64.1, 58.3, 50.1 and 38.2 kD). An immunoblotting assay with anti-*L. dispar* lipophorin antiserum proved negative results (data not shown).

DISCUSSION

Earlier studies of the cabbage moth by Sass (1988) demonstrated by Coomassie brilliant blue R-250 staining (in figure after SDS-PAGE) that the isolated storage protein is composed of two subunits, the R_f values of which are lower than that of BSA (68 kD), but higher than that of phosphorylase B (97 kD). In our electrophoresis system, these subunits ran at 81.9 and 74.6 kD. The gels were stained with silver in this study, and seven types of subunits were revealed in the cabbage moth storage protein: two dominant subunits with molecular masses of 81.9 and 74.6 kD, and five minor ones with molecular masses of 64.1, 58.3, 50.1, 40.8 and 38.2 kD. It is interesting that the molecular weights of certain subunits (at 58.3, 50.1 and 38.2 kD) are very similar to the MW of proteins whose phosphorylation in the cabbage moth fat body was induced by 20-hydroxyecdysone (Sass, 1988), and which had molecular masses of approx. 58, 50 and 38 kD. It is well-known that the fat body is the primary site for the synthesis of storage proteins, secreting them as glycosylated hexa- or octomeric aggregates composed of 70,000- to 90,000- M_r subunits (Miller & Silhacek, 1982a,b; Levenbook, 1985).

As mentioned in the introduction, most lepidopteran storage proteins are composed of one or two types of subunits. Two types of cabbage moth storage protein subunits (M_r 81.9 and 74.6 kD) were accordingly observed after staining SDS-denaturing gels with Coomassie brilliant blue (data not shown). Staining with silver, however, revealed five further minor subunits. According to Levenbook (1985), an unusual feature of larval haemolymph protein-I (*Calliphora vicina*) is its instability when boiled in SDS. Lower M_r break-down products begin to form after heating the protein for 4–5 min in 1% (w/v) SDS at 100°C, as was done in this study.

The presence of minor subunits was confirmed by immunoblots with monospecific anti-*L. dispar* arylphorin. Similarly, Riddiford & Hice (1985) noticed that immunoblots of *M. sexta* revealed the presence of arylphorin; additionally, several small polypeptides, which cross-reacted with the arylphorin antibody, were found in the fat body only shortly before

and after larval ecdysis. These authors suggest that the storage protein might be broken down in the fat body, and then used during the moult for cuticle production or other types of protein synthesis. In our study, the number of minor subunits was not uniform during the observed period of cabbage moth development. As seen after immunoblotting (Fig. 9b), the number was comparatively lower in 5th instar larvae, and especially after moulting. It is probable that these subunits, which were relatively weakly bound (the storage protein being dissociated into subunits in non-denaturing isoelectrofocusing), were used as sources of amino acids during moulting.

Many authors (Tojo et al., 1978; 1980; 1981; Miller & Silhacek, 1982 b; Telfer et al., 1983; Bean & Silhacek, 1989) have noted that, in lepidopterans, storage proteins are produced by the fat body during the final larval instar. These proteins are secreted into the haemolymph during the feeding stage, then taken back up into the fat body during the prepupal stage, where they are sequestered into storage granules. In the present study, the results after SDS-PAGE and immunoblotting (Fig. 9) indicated that the storage protein reached its highest level in haemolymph serum of cabbage moth prepupae and pupae on day 1. This amount decreased in pupae on day 3.

Generally, insect storage proteins are acidic (Roberts & Brock, 1981), although there exists some evidence concerning pIs which are almost neutral (Kim et al., 1989). The pIs of cabbage moth storage protein subunits (from 5.4 to 6.3) are similar to those of other insect species, and are somewhat more acidic than the pH (6.76) of the 6th instar larvae haemolymph (unpublished data). According to Munn et al. (1971), however, the pH of the haemolymph of *Calliphora erythrocephala* changes from approx. 6 during the larval instar to approx. 7 during the pupal stage.

The cabbage moth storage protein was dissociated into at least 7 subunits, depending upon the pH of the buffers. The experiments indicated that larval haemolymph proteins are a pH-sensitive associating-dissociating system (see Levenbook, 1985). Up to pH 10.5, minor conformational changes are reversible by lowering the pH, whereas above pH 10.5 the loss of a secondary structure becomes irreversible and the protein starts to denature. Levenbook (1985) stated that the pH of the PAGE buffers was demonstratably critical for conformational changes. This means that the pH stability of the cabbage moth storage protein (which is an ampholyte; unpublished data) should also be observed in various PAGE buffers. Other storage proteins [manducin of *Manduca sexta* (Kramer et al., 1980) or the protein-6 of *R. americana* (de Bianchi & Marinotti, 1984)], however, do not become dissociated at pH as high as 9.0, thus indicating the diversity of storage proteins in various species of insects.

One of the features of the cabbage moth storage protein was its conjugation with lipo- and glyco- components. The conjugation of storage proteins with lipo-components is characteristic of lipophorins (e.g. *Blatta orientalis*: Duhamel & Kunkel, 1983). Glyco-components are bound to most arylphorins (e.g. *R. americana*: de Bianchi & Marinotti, 1984). The binding of both components was, however, observed in the larval haemolymph protein of *Locusta migratoria* (de Kort & Koopmanschap, 1987), and is often characteristic of native chromolipoproteins (Jones et al., 1988). Although the cabbage moth storage protein contained a lipo-component, it reacted with antiserum to the arylphorin of *L. dispar*. Immunoblotting of this protein with antibodies raised against *L. dispar* lipophorin indicated no antigenic cross reactivity. In spite of the fact that a lack of cross-reactivity was

shown for apoLp-I from the lipoproteins of several insects (Fernando-Warnakulasuriya & Wells, 1988), and that arylphorins show immunological relationships within and among species (Tsakas et al., 1991), our data indicate that the *M. brassicae* storage protein belongs to the class of storage proteins presently known as arylphorins.

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