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On the Occurrence and Distribution of Storage Proteins During the Metamorphosis of *Bombyx mori* L.

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누에의 變態에 따른 貯藏蛋白質의 出現과 分布에 관하여

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摘 要

누에의 變態期 동안 貯藏蛋白質의 出現과 번데기 시기동안 각 組織에 따른 貯藏蛋白質의 分布를 살펴보기 위해 電氣泳動法, 免疫學的 方法 및 column chromatography法을 使用하였다.

혈림프의 貯藏蛋白質은 2개로 區分이 되고, 5령 初期부터 出現하고 있으며 지방체 단백질과도 동일한 전기영동상의 移動度를 갖고 있다. 이의 量的 變化는 終令期에는 혈림프에서 높은 濃度를 유지하다. 蛹化後에는 脂肪體에 축적이 되는 경향을 나타내며 특히 저장단백질-2가 암수에서 모두 두드러진 저장단백질의 양상을 보이고 있다. 이렇한 貯藏蛋白質은 종령 末期에는 큐티클 단백질 형성에도 관여하는 것 같으며 번데기에는 中腸도 일시적으로 저장단백질을 저장하는 것 같다. 또한 貯藏蛋白質中 저장단백질-2는 vitellogenin과 전기영동 및 면역학적으로 동일한 이동도를 나타내고 있으며 특히 번데기시기동안 卵黃蛋白質의 항체에 대해 항원-항체반응을 나타내고 있어 卵形成過程에도 밀접하게 관여하는 것 같다.

INTRODUCTION

Fat body of the insect is considered to be equivalent to the liver of vertebrate in viewpoint of the center of intermedial metabolism and the synthesis of proteins in haemolymph. During last larval-pupal edysis, however, the fat body is known to play a great role in storing materials for the formation of adult tissue (Price, 1973). It has been proved by electrophoresis and immunological methods that these proteins are selectively absorbed from haemolymph into fat body (Chippendale and Kilby, 1969; Collins and Downe, 1970;

Chippendale, 1970; Martin *et al.*, 1971). Also, Deposition of haemolymph proteins in fat body during metamorphosis of larvae to pupae was reported in *Calpodes ethlius* (Locke and Collins, 1968) and *Pieris brassicae* (Chippendale and Kilby, 1969). Kinnear and Thomson (1975) reported with *Callphora* that calliphorin, the haemolymph storage protein is synthesized in fat body of mature larvae, occupying 60% of total proteins in haemolymph but decreases in concentration due to the deposition in fat body during pupal stage while Tojo *et al.* (1978) indicated with *Hyalophora cecropia* that two kinds of storage proteins were separated in fat bodies and these proteins begin to increase in haemolymph during larval instar and reaches the peak during spinning period but decrease thereafter due to the deposition in fat body during pupal stage.

Althongh there were so many studies about quantitative change in storage protein, little is known about the distribution of these proteins. The present study was undertaken to determine the contribution of storage proteins to adult tissue and organ formation by tracing the occurrence and distribution of storage proteins in various organs during pupal stage.

MATERIALS AND METHODS

1. Insects

Fourth instar *Bombyx mori* were obtained from Sericultural Experiment Station at Suweon and reared on mulberry leaves at the temperature of 25±2°C and the relative humidity of 70~80%. Male and female were used at the stages of 2, 4, 6, 8 day old last instar larvae, prepupae, and 1, 3, 5, 7, 9 day old pupae.

2. Extraction of protein samples

1) Extraction of haemolymph

Last instar larvae and pupae were rinsed in distilled water and each haemolymph was collected in test tube on ice by puncturing the abdomen with sharp needle. A small amount of phenylthiourea was added to haemolymph in order to prevent darkening and the haemolymph was centrifuged at 10,000 rpm for 10 min and stored at −70°C until used.

2) Extraction of proteins from fat body, midgut, cuticle, silk gland, and ovary.

Haemolymph removed *Bombyx mori* was dissected out in cold ringer solution. Fat body, midgut, cutcle, silk gland, and ovary each was weighed and collected in test tube and homogenized, centrifuged at 10,000 rpm for 10 min and then their supernatants were filtered through 4 layers of cotton gauzes to remove fat and used as protein sample.

3. Electrophoresis

Electrophoresis was carried out in 5% and 7.5% polyacrylamide disc gels with Tris-glycine buffer (pH 8.3) according to the method of Davis (1964). After running gels, each gel was stained with Coomassie brilliant blue R-250 (0.25%) and scanned with densitometer (TG 2970).

4. Immunological methods

1) Immunodiffusion

8 day old female last instar larval haemolymph and purified storage protein were used as antigen and antibody was prepared by injecting mixture solution of 0.5 ml sample and 0.5 ml complete freund's adjuvant into rabbit subcutaneously several times at the intervals of one week. Immunodiffusion was carried out mostly according to the method of Ouchterlony (1949). 1% agarose gel was made in 0.01 M phosphate buffer (pH 7.0) including 0.15 M Nacl and 0.1% sodium azide and diffusion was conducted in room temperature for 3 days and rinsed in 0.15 M Nacl solution to remove unreacted antibodies and antigens and stained in 1% amido black 10B.

2) Immunoelectrophoresis

1% agarose gel was made in 0.01 M veronal buffer (pH 8.6) including 0.1% sodium azide. Electrophoresis was run in 0.01 M veronal buffer (pH 8.6) at 150 v for 1 ½ hrs and left at room temperature for 1 day after addition of antibody and stained.

5. Purification of storge protein

Storage protein was purified according to the method of Tojo *et al.* (1980). Female pupal fat bodies were dissected out and homogenized in 0.05 M phosphate buffer (pH 7.5) and centrifuged at 10,000 rpm for 20 min and the supernatant was filtered through cotton gauzes. Ammonium sulphate, which stabilizes proteins, was added to this extract up to a concentration of 5% saturation and pH was adjusted to 7.5 with 0.01 M NaOH. 10ml of this sample is put into the test tube and boiled in 76°C for 10 min and centrifuged at 10,000 rpm for 20 min and the supernatant was used as sample. Storage protein-1 of the supernatant was obtained through column chromatography from precipitate by 20~40% saturation with ammonium sulphate whereas storage protein-2 from precipitate by 40~60% saturation with ammonium sulphate.

RESULTS

Present experiment was to focus on the quantitative change of storage protein in haemolymph and deposition of storage protein in fat body during the period of last instar larval to pupal stage and the distribution of stoage protein and also relationship with yolk protein during pupal stage.

In the haemolymph of male and female, two storage proteins designated SP-1 and SP-2 appeared in the upper region of the gel. SP-1 and SP-2 showed a quantitative change in haemolymph and fat body of male and female during metamorphosis (Fig. 1, 2). The quantitative change of SP-1 and SP-2 were compared using densitometer. Major change was that SP-1 in female showed a drastic decrease in haemolymph after last larval stage whereas a distinct accumulation in fat body after pupation (Fig. 3). However, there occurred no noticeable change in male haemolymph and fat body. On the while, storage

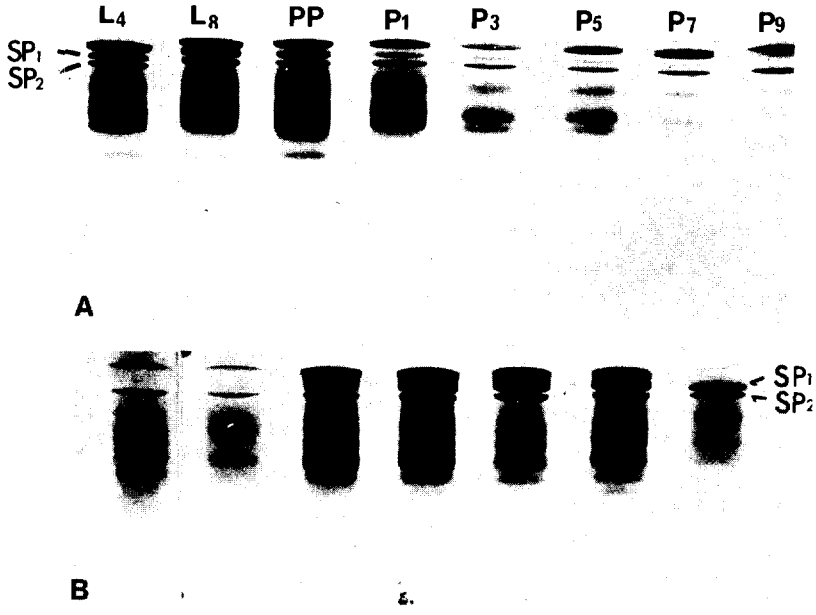

Fig. 1. Electrophoretic patterns of haemolymph and fat body in female *Bombyx mori* during the metamorphosis. (A) female haemolymph; (B), female fat body; SP_1; storage protein-1; SP_2, storage protein-2.

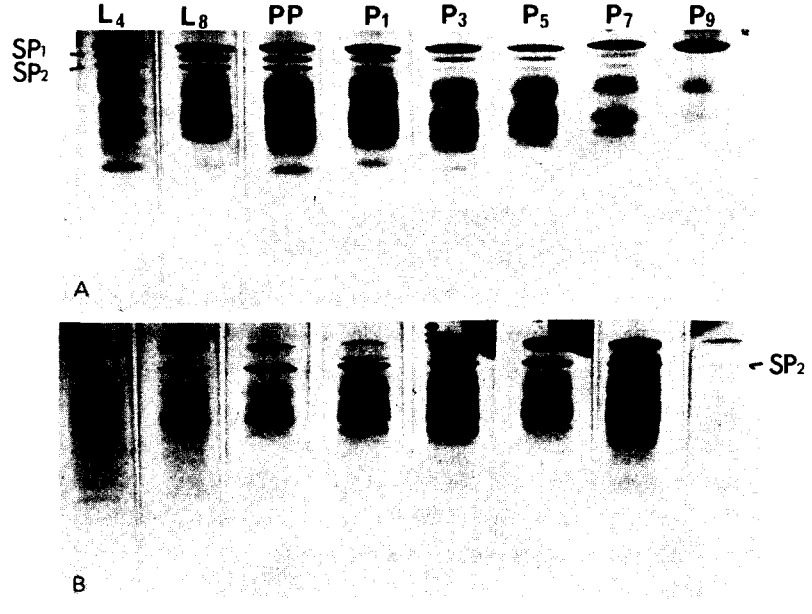

Fig. 2. Electrophoretic patterns of haemolymph and fat body in male *Bombyx mori* during the metamorphosis. (A) male haemolymph; (B) male fat body; SP_1, storage protein-1; SP_2, storage protein-2.

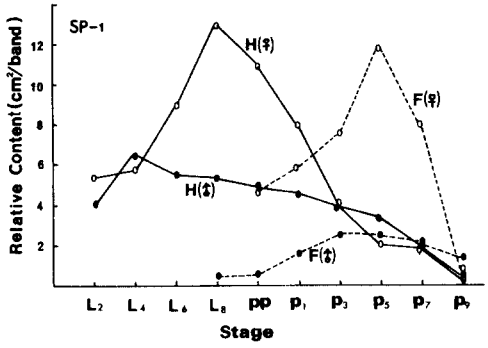

Fig. 3. Changes of relative content of storage protein-1 in male and female haemolymph and fat body during the metamorphosis.

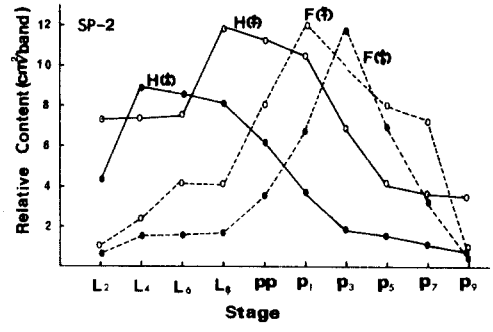

Fig. 4. Changes of relative content of storage protein-2 in male and female haemolymph and fat body during the metamorphosis.

protein-2 revealed more deposition in fat body than that of storage protein-1 (Fig. 4).

To trace the distribution of storage proteins, antibody against 8 day old female last instar larval haemolymph was prepared and used for immunoelectophoresis. About 4 to 5 precipitin

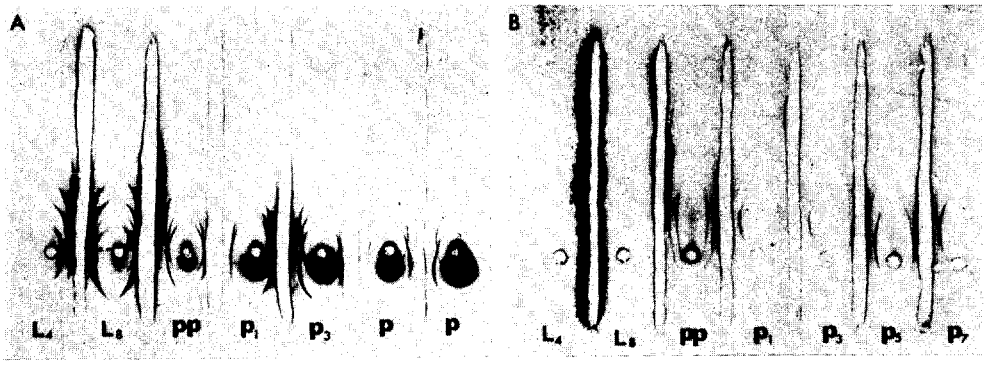

Fig. 5. Immunoelectrophoretic patterns of female haemolymph and fat body during the metamorphosis. (A) female haemolymph; (B) female fat body.

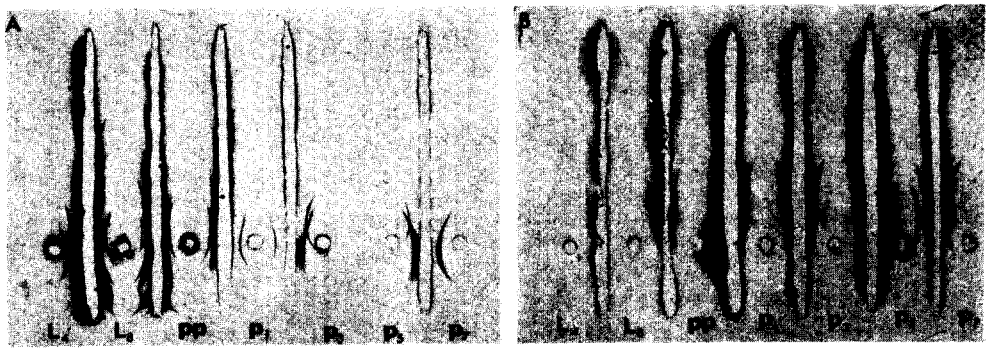

Fig. 6. Immunoelectrophoretic patterns of male haemolymph and fat body during the metamorphosis. (A) male haemolymph; (B) male fat body.

arcs appeared in the haemolymph of female last instar larvae but fewer arcs in pupal stage (Fig. 5A). In the fat body only precipitin arc for SP-2 was confirmed after pupation (Fig. 5B). On the while precipitin arc for SP-2 scarcely appeared in male haemolymph (Fig. 6A) and also arc for SP-1 did not appear in fat body after pupation but SP-2 showed a sizable accumulation in fat body (Fig. 6B). There was a drastic accumulation of SP-2 rather than SP-1 in fat body during the last larval-pupal transformation.

These storage protein-1 and-2 each was purified according to the method of Tojo *et al.* (1980). SP-1 was precipitated from protein sample between 20 and 40% saturation with $(NH_4)_2SO_4$ and chromatographed on a column of CM-Sephadex and DEAE-Sephadex and concentrated by freeze dryer (Fig. 7). On the while SP-2 was precipitated between 40~60% saturation with $(NH_4)_2SO_4$, followed by CM-cellulose and DEAE-cellulose (Fig. 8).

Female specific protein, vitellogenin was run with storage proteins, showing that vitellogenin is almost identical to SP-2 in mobility level of band (Fig. 9A) and also to precipitin arc for SP-2 in immunoelectrophoresis (Fig. 9B).

The distribution of haemolymph proteins in various tissues was determined by immunodiffusion method using antibody against the haemolymph of 8 day old last instar larvae. The reaction with haemolymph protein showed more precipitin lines in pupal stage than in last larval instar stage in fat body with 3 to 4 lines in female while 2 to 3 lines in male (Fig. 10b, d). This pattern demonstrates that haemolymph protein is gradually accumulated

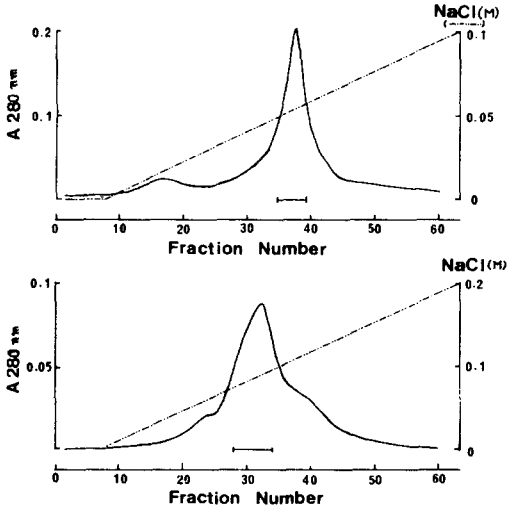

Fig. 7. Column chromatographic separation of storage protein-1 from fat body, The proteins precipitated by $(NH_4)_2SO_4$ between 20 and 40% saturation were fractionated by column chromatography on CM-sephadex (upper) and DEAE-sephadex (Lower).

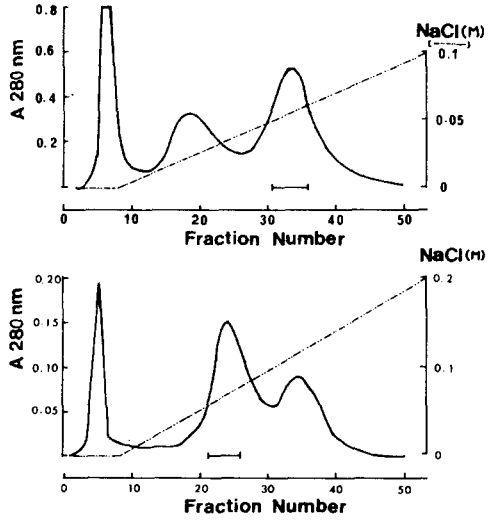

Fig. 8. Column chromatographic separation of storage protein-2 from fat body. The proteins precipitated by $(NH_4)_2SO_4$ between 40 and 60% saturation were fractionated by column chromatography on CM-cellulose (upper) and DEAE-cellulose (lower).

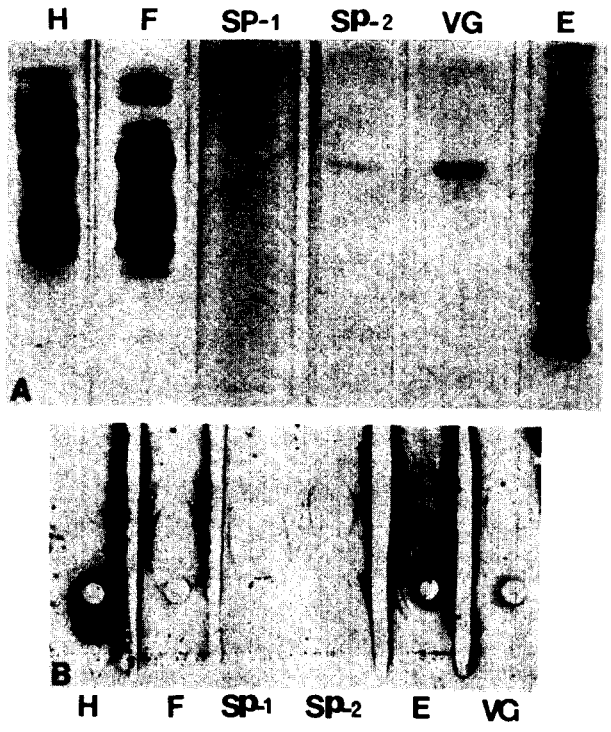

Fig. 9. A) Electrophoretic patterns of purified storage protein-1 and storage protein-2 and B) Immunoelectrophoresis patterns of purified storage protein-1 and storage protein-2. Letter symbols indicate; H, haemolymph; F, fat body; SP-1, storage protein-1; SP-2, storage protein-2; VG, vitellogenin; E, egg.

into fat body during pupal period. The reaction with haemolymph protein showed more precipitin lines in last larval instar stage than in pupal stage (Fig. 10a, c). Also, in the midgut there were two precipitin lines in last larval instar stage but 3 lines in 1 day old pupae and 4 lines in 3 day old pupae, demonstrating that midgut contains part of haemolymph proteins during pupal stage (Fig. 11a). Also, antibodies against haemolymph proteins showed 2 to 3 precipitin lines with silk gland protein and 3 lines with that of 1 day old pupae (Fig. 11b). There are two precipitin lines with cuticle proteins until early pupal stage but no reaction after 3 day old pupae (Fig. 11c).

Also, antibody against purified storage protein-2 was prepared and used to determine its distribution in various organs. SP-2 is present in low concentration in male and female haemolymph during the pupal stage (Fig. 12a). In fat body, however, SP-2 is absent in last larval instar stage but present in high concentration after pupation (Fig. 12b). This SP-2 showed a clear positive reaction with haemolymph and fat body but different rections with midgut and cuticle during development stage. There was a positive reaction with midgut in 3 day old pupae and with cuticle in newly ecdysed pupae but no reactions with

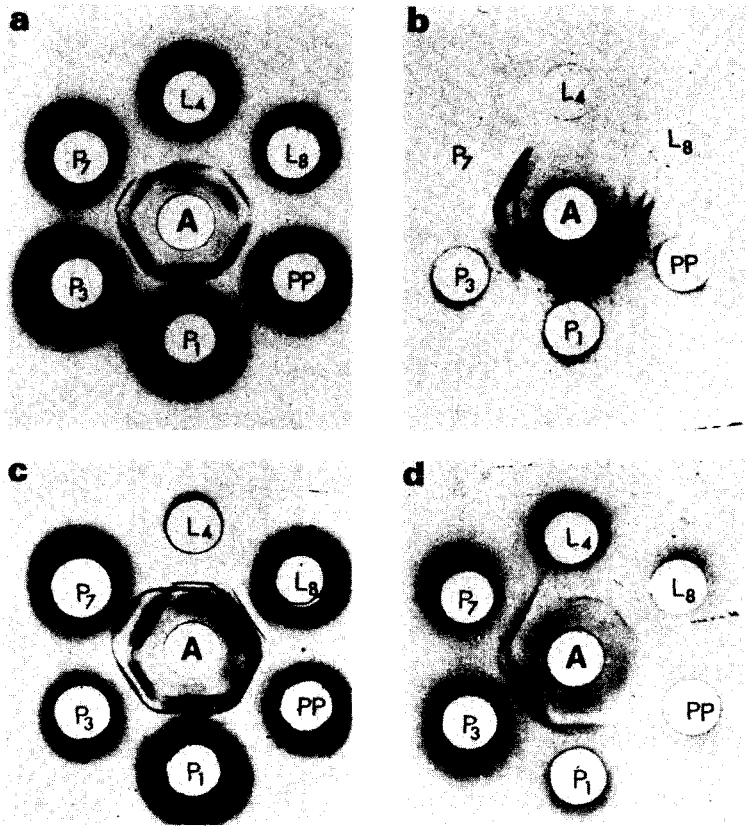

Fig. 10. Double diffusion precipitation pattern against female haemolymph during larval and pupal stage. a) female haemolymph; b) female fat body, c) male haemolymph, d) male fat body. Stages; L_4, 4 day larva; L_8, 8 day larva; PP, prepupa; P_1, 1 day pupa; P_3, 3 day pupa; P_7, 7 day pupa.

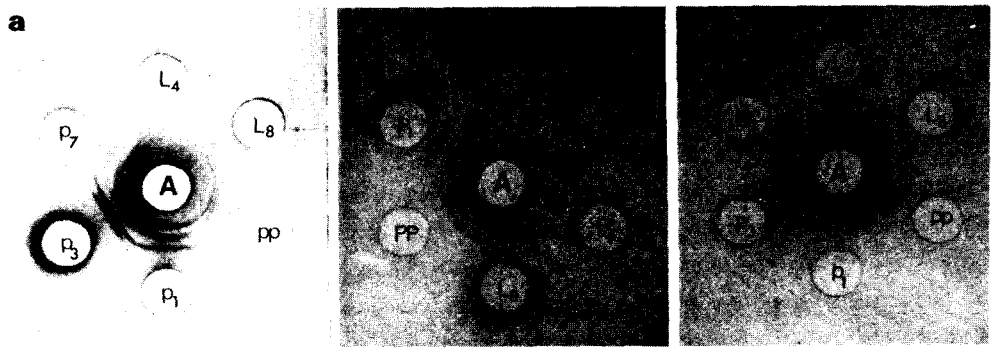

Fig. 11. Immunodiffusion patterns against female haemolymph during larval and pupal stage. a) midgut, b) silk gland, c) cuticle.

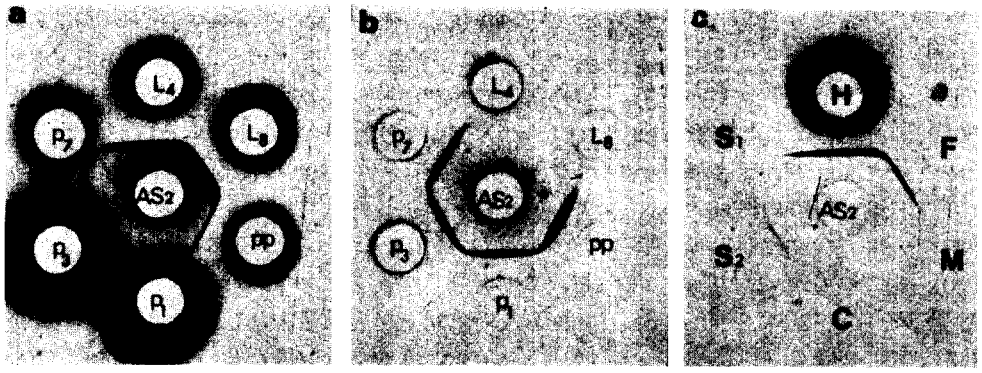

Fig. 12. Immunodiffusion patterns against storage protein-2 (AS_2) during the metamorphosis. a) haemolymph, b) fat body, c) H, haemolymph; F, fat body, M, midgut; C, cuticle; S_1 storage protein-1; S_2, storage protein-2.

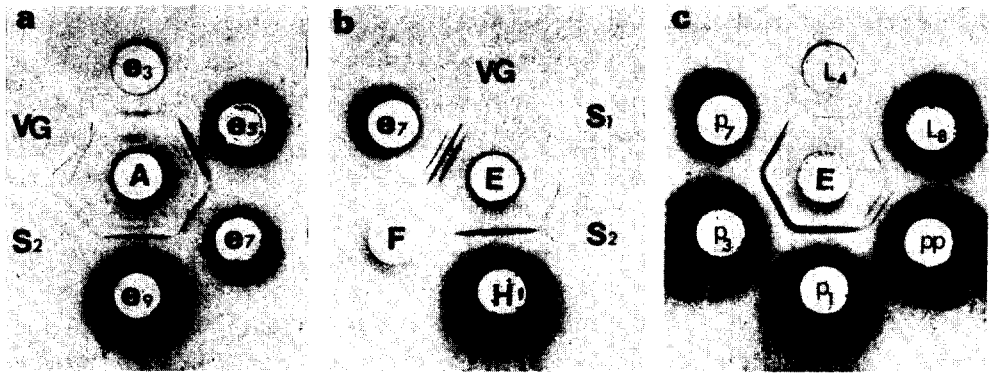

Fig. 13. Immunodiffusion patterns against haemolymph (A) and egg (E) protein during the larval and pupal stage. a) egg protein, vitellogenin (VG), and storage protein-2 (S_2); b) VG, storage protein-1, storage protein-2, haemolymph, fat body and egg c) Haemolymph protein.

midgut and cuticle in prepupal stage (Fig. 12c). Also, haemolymph protein showed a homogenity with yolk protein, especially vitellogenin and SP-2 represented homogenity with haemolymph protein, demonstrating that they might be involved in egg formation (Fig. 13a). The involvement of haemolymph protein for yolk protein appear to be greater in pupal stage than in last larval instar stage (Fig. 13c) and vitellogenin and SP-2 showed same precipitin line with yolk protein (Fig. 13b).

DISCUSSION

The fact that storage proteins and fat body proteins have identical electrophoretic mobility since storage proteins are primarily synthesized in the fat body has been proved with *Calliphora erythrocephala* (Munn and Greville, 1969; Kinnear *et al.*, 1971) and *Calpodes*

ethlius (Collins, 1975). In the present experiment storage proteins of haemolymph consist of two proteins which have identical electrophoretic mobilities with equivalent fat body proteins. These storage proteins maintain high concentration during last larval instar stage but gradually accumulate in fat body after pupation. Kinnear and Thomson(1975) found with *Calliphora stygia* that when labelled haemolymph proteins are injected into larvae, these proteins accumulated in the fat body. This accumulation has been observed in relation to the decreasing concentration of haemolymph storage proteins during the metamorphosis (Kramer *et al.*, 1980; Tojo *et al.*, 1978). Locke and Collins(1968) reported that several major haemolymph proteins disappear with the simultaneous deposition of granular proteins in fat body of *C. ethlius*. In the present experiment storage proteins of *Bombyx mori* showed differential pattern to some extent for accumulation between sex. Two storage proteins of female haemolymph are all stored in fat body after metamorphosis but in the case of male, storage protein-1 showed a slight decrease in haemolymph with the simultaneously slight increase in fat body, indicating that storage protein-2 in both sexes plays a greater role in storage protein. Especially SP-2 is almost similar to female specific protein, vitellogenin in electrophoresis and immunoelectrophoresis, suggesting that SP-2 is involved in the formation of yolk protein. According to Kawaguchi and Doire (1973) using acrylamide gel electrophorsis for analysis of *Bombyx* haemolymph, since female specific larval protein disappears just before pupation and new female specific protein appears after pupation, female larval protein is regarded as storage protein-1 while pupal protein as vitellogenin. In the present work storage protein-2 is believed to be vitellogenin. These storage proteins are involved in the synthesis of adult tissue. In fact, in Diptera storage protein, calliphorin is high in phenylalanine and tyrosine (Munn *et al.*, 1971; Kinnear and Thomson, 1975) and also lucilin has similar composition (Thomson *et al.*, 1976), suggesting that these proteins are utilized in the formation of cuticle protein. In the present experiment the antibodies against haemolymph proteins showed 3 precipitin lines with cuticle proteins in late last larval and prepupal stages, indicating that haemolymph proteins are directly transported to the cuticle. Especially midgut protein showed 2 precipitin lines until prepupal stage and another 1 or 2 new precipitin lines from 1 day old pupae, suggesting that midgut appears to have storage capacity during metamorphosis. However, little is known about storage function of midgut, although Wyatt(1958) reported with *Hyalophora cecropia* that midgut temperally stores haemolymph proteins during the period of diapause.

The antibodies against haemolymph proteins showed 1 or 2 lines with fat body proteins in last larval instar stage and 3 to 5 lines after pupation and 1 or 2 lines with newly formed yolk proteins after pupation. Antibodies against yolk proteins showed 1 line with haemolymph protein in last larval instar stage and 2 lines after pupation, indicating that new haemolymph proteins are involved in egg formation. As to the origin of haemolymph protein, Latfer(1960) reported that fat body is major source of haemolymph proteins based on the mobility of starch gel electrophoresis and also Price(1973) pointed out that fat body

synthesize most of haemolymph proteins, but Boavida and Roberts(1975) reported that most of haemolymph proteins are synthesized in fat body but some haemolymph proteins are formed in haemocyte, Malpighean tubule, and salivary gland. In the present work, it was found that fat body synthesizes most of haemolymph proteins during last larval instar stage but stores some haemolymph proteins after pupation.

SUMMARY

Electrophoretic, immunological, and column chromatography methods were used to determine the appearance and distribution of storage proteins in various organs during the metamorphosis of *Bombyx mori* L.

Two storage proteins start to appear in haemolymph in early 5th instar stage and show the identical mobility with fat body proteins. These proteins show the high concentration in haemolymph in last instar stage but accumulate in fat body after pupation. Storage protein-2 shows the distinct pattern for general storage proteins in both male and females. This protein is involved with the formation of cuticle protein in late last instar stage and appears to be temperally deposited in midgut during the pupal stage. Also SP-2 shows the identity with vitellogenin electrophoretically and immunologically and especially the positive reaction with antibody against yolk protein during the pupal stage, demonstrating that the storage protein is closely related to the formation of yolk protein.

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