Quantitative Changes of Storage Proteins and Vitellogenin during the Pupal-Adult Development in the Silkworm, *Bombyx mori* (Lepidoptera : Bombycidae)

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(Received February 19, 1981)

Accumulation and distribution of two storage proteins, SP-1 (female-specific) and SP-2, and vitellogenin in the silkworm, *Bombyx mori*, were studied during the pupal period by means of Oudin's single diffusion tests using antisera specific to these proteins. In females SP-1 and SP-2 account for approximately one fourth of the total protein in the newly ecdysed pupa, while in males which lack SP-1, SP-2 comprises one twentieth of the total protein. Both proteins decrease slightly in the early half of the pupal stage, and then rapidly decline so as to disappear shortly before adult emergence. During the metamorphosis, most of them are found in the fat body, negligible amounts in the haemolymph and essentially none in other tissues. The content of vitellogenin is 5% of the total protein in the newly pupated female, but increases gradually in the early pupal stage, and then during pharate adult development it accumulates sharply up to several times the initial amount, attaining to 40% of the total protein in the newly ecdysed adult. In the early half of the pupal stage, vitellogenin is detectable only in the haemolymph, but in the latter half it increases rapidly in the ovary while maintaining its level in the haemolymph. The amino acid composition of vitellogenin is similar to that of SP-1 except that the former is higher in glutamate and serine, but lacks the exceptionally high methionine of the latter.

INTRODUCTION

In holometabolous insects, the radical transformation from larval to adult form takes place within the pupa, which is a closed system except for gaseous exchange. Hence, the raw materials for adult tissue formation must be supplied internally partly from components of degenerating larval tissues. It has been generally accepted, but with little evidence, that another major source of materials for histogenesis is the fat body cells which hypertrophy and become loaded with various kinds of storage organelles including protein granules during larval-pupal ecdysis (PRICE, 1973; THOMSON, 1975).

Recently, Tojo et al. (1978) identified two storage proteins in protein granules isolated from the fat body of the saturniid moth, *Hyalophora cecropia*. Both proteins

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accumulate in the haemolymph of the mid-final instar larva, reach maximal levels at spinning, and then are sequestered into fat body so as to make up 60% of the total fat body protein in the female pupa. Although these proteins exist in both sexes, the levels of them in the fat body are two or three times higher in females than in males. Further, Tojo et al. (1980) showed that two storage proteins named SP-1 (female-specific) and SP-2 in the silkworm, *Bombyx mori*, occur in the haemolymph of the last instar larva similarly to the *Cecropia* storage proteins. At pupation, they move almost entirely into the fat body attaining to 60% of the total fat body protein in females, while in males which lack SP-1, SP-2 comprises 20% of the total fat body protein.

In female *B. mori*, another female-specific protein originally named FP and now recognized as vitellogenin, a precursor of yolk protein, appears in the haemolymph of the newly ecdysed pupa, and accumulates in the ovary during pharate adult development (KAWAGUCHI and DOIRA, 1973). The pupal fat body has an ability to synthesize vitellogenin (ONO et al., 1975), which is consistent with a finding that the fat body mRNA from the last instar larva directs the synthesis of storage proteins, whereas that from the female pupa encodes vitellogenin in a wheat germ system and in *Xenopus* oocytes (IZUMI et al., 1980b). Also in *H. cecropia* vitellogenin is first synthesized by the fat body shortly after spinning (PAN et al., 1969; PAN, 1971), and increases in the haemolymph, attaining to a maximal level at pupation (TELFER, 1954). The above two silkworms deposit yolk mostly during pharate adult development and complete oogenesis before emergence, which implies that the females have some internal reserve source for vitellogenin synthesis. Storage proteins are a possible candidate for this source.

In this study, we have tried to show some relationships between storage proteins and vitellogenin by comparing developmental profiles of these proteins in the whole body and in some tissues during the pupal period of the silkworm, *Bombyx mori*.

MATERIALS AND METHODS

Insects. Silkworm larvae of N124×C124 hybrid and E20 strain were reared on mulberry leaves, and the pupae were kept at 25°C until used.

Dissection of tissues. Six to eight animals were taken at two-day intervals from pupation to emergence. The anal margin of wings was punctured with a needle, gentle pressure was applied to the abdomen, and the haemolymph was collected. It was centrifuged at 7,000 g for 3 min at 4°C, and the supernatant was diluted eight-fold with cold buffered saline (0.01 M sodium phosphate buffer-0.14 M NaCl, pH 7.0 with a few crystals of phenylthiourea). The pupa was then opened under buffered saline, and the alimentary canal, Malpighian tubes and testes were removed. The fat body and ovary were taken out and washed with saline. The remaining body wall was designated as the integument, although it included muscles and the imaginal organs such as wings and legs together with the integument. The separated tissues and haemolymph were placed in tubes and kept at -20°C until analysis.

The volume of pupal haemolymph was calculated, assuming that the haemolymph occupied one fourth of the body weight, according to the result by HORIE et al. (1971).

Extraction of proteins. One whole animal or tissue fraction (except haemolymph)

was homogenized with three volumes of buffered saline in a glass homogenizer and centrifuged at 2,000 *g* for 20 min at 4°C. The residue was extracted with saline further four times. The supernatants were combined, diluted to a definite volume, and stored at -20°C until analysis.

In case of whole animals, the residue after the extraction of soluble protein was washed twice with each of cold 5% perchloric acid, 95% ethanol, ethanol-ether (3 : 1, v/v) and ether. From the final residue, insoluble protein was extracted with hot 0.25 M NaOH. Proteins in the soluble and insoluble fractions were determined by the method of LOWRY et al. (1951), with calf serum albumin as the standard.

Preparation of specific antisera. To prepare antisera against SP-1 or SP-2, the respective proteins purified as described by Tojo et al. (1980) were dissolved in saline at 5 mg/ml concentration, 0.5 ml portions were mixed with equal volumes of Freund's complete adjuvant, and injected into the foot pads of rabbits. Two additional injections were given subcutaneously at weekly intervals. The rabbits were bled two weeks after the final injection and the sera were stored at -20°C.

To prepare antiserum against vitellogenin, 0.5 ml portions of the extract from ovaries of the newly emerged female with ten volumes of saline were injected into a rabbit as described above. The serum obtained was mixed with male pupal haemolymph and centrifuged to remove antibodies not specific to the female, as described by TELFER (1954).

Immunodiffusion analysis. Concentrations of SP-1, SP-2 and vitellogenin were determined by the quantitative immunodiffusion technique of Oudin as described by TELFER and WILLIAMS (1953), using the respective purified proteins as standards. Vitellogenin was purified from haemolymph by Dr. O. YAMASHITA according to the method described by CHINO et al. (1969) for "DGLP II", which was later identified as vitellogenin (CHINO et al., 1976). This was also used for amino acid analysis.

RESULTS

Changes in the contents of proteins in the whole insect

In the newly pupated females of E20 strain, SP-1 and SP-2 together amounted to 23 mg per insect or 19 mg/g weight, while in males, SP-2 content was 3 mg per insect, or 3 mg/g (Fig. 1). These values corresponded to 23 and 4% of the total protein, respectively. Both storage proteins changed similarly during the pupal period; they decreased gradually in the early half, and then declined rapidly finally disappearing before adult emergence. Vitellogenin in females, on the other hand, first increased gradually from 5 mg per insect (5% of the total protein), and then during pharate adult development increased sharply, attaining 28 mg (40% of the total protein) in the newly emerged adult.

In the case of N124×C124 hybrid, the total amounts of soluble and insoluble proteins were determined in addition to the storage proteins (Fig. 2). The patterns of change in SP-1 and SP-2 contents in females were similar to those observed in the E20 strain. But, the initial content of SP-2 in N124×C124 was double that in E20, and SP-1 and SP-2 together amounted to 34 mg per insect, or 26% of the total protein. The content of the total protein remained rather constant, but shortly before emergence decreased sharply, which mostly due to a decline in the soluble fraction. As indicated in Fig. 2, vitellogenin accumulated up to 30 mg in the adult female, which

Fig. 1. Changes in the contents of SP-1, SP-2 and vitellogenin during pupal period of the silkworm. Strain, E20. Weight of newly ecdysed pupa, 1.23 g for females and 0.96 g for males. Each point is the mean of six to eight replicates with S. E. M.

Fig. 2. Changes in the contents of soluble and insoluble proteins, SP-1 and SP-2 during pupal period of the female silkworm. Hybrid, N124 x C124. Arrow indicates the level of vitellogenin in the newly ecdysed adult. Weight of newly ecdysed pupa, 1.42 g. Each point is the mean of four to five replicates with S. E. M.

Fig. 3. Changes in the contents of SP-1, SP-2 and vitellogenin in different tissues during pupal period of the silkworm. Hybrid, N124 x C124. O, Fat body; Δ , haemolymph; $\bullet$, integument; $\square$, ovary. Left figure: solid line, SP-1; dotted line, SP-2. Each point is the mean of six to eight replicates with S. E. M.

represented 43% of the total protein.

Distribution of storage proteins and vitellogenin in tissues

As shown in Fig. 3, in female of N124 x C124, SP-1 and SP-2 were found mostly in the fat body and declined in the tissue of the mid-pupal stage when the histolysis of the fat body proceeded. Slight increases in SP-1 and SP-2 contents were observed in the haemolymph of this stage, amounting to 1% of the content in the whole insect,

Table 1. AMINO ACID COMPOSITION OF VITELLOGENIN FROM SILKWORM OVARY^a

Amino acid	Moles/1000 moles determined amino acids
Cysteine	Trace
Aspartate	116
Threonine	60
Serine	85
Glutamate	137
Proline	45
Glycine	57
Alanine	53
Valine	65
Methionine	14
Isoleucine	56
Leucine	80
Tyrosine	43
Phenylalanine	44
Lysine	74
Histidine	28
Arginine	43
Total	1000

^a Protein was hydrolyzed in 6 M HCl at 110 °C for 24, 48 and 72 hr, and amino acids determined with a JEOL 6AH analyzer.

but essentially none was found in other tissues. Figure 3 also shows that vitellogenin was distributed only in the haemolymph during the early half of the pupal period, but then increased rapidly in the ovary during yolk deposition, while the level in the haemolymph remained constant.

Amino acid composition of vitellogenin

As given in Table 1, vitellogenin was rich in aspartate and glutamate, high in serine, valine, leucine and lysine, poor in methionine and almost lacks cysteine.

DISCUSSION

Immunological study on the quantitative change of the protein, which is believed to have storage role, during metamorphosis of insects has hitherto been reported only in *Drosophila melanogaster* with two larval serum proteins called LSP-1 and LSP-2 (ROBERTS et al., 1977). LSP-1 and LSP-2 represent 9 and 1.5% of the total soluble protein at puparium formation, but they decrease rapidly during the later half of metamorphosis, falling to 2 and 0.5% of the total soluble protein at emergence, respectively, and disappear within further three days. Electrophoretic studies showed that in *Calliphora erythrocephala*, calliphorin content accounts for 60% of the total soluble protein at the maximum, but drops to 10% at emergence (MUNN and GREVILLE, 1969), and in *C. stygia*, the content falls from 3 mg per insect at pupation to 0.03 mg at emergence (KINNEAR and THOMSON, 1975).

These developmental profiles of dipteran proteins are similar to those of the two storage proteins, SP-1 and SP-2 of *B. mori* studied in this article, except that in the latter species both disappear in the whole insect before adult emergence. SP-1 and

SP-2 are found mostly in the fat body, but essentially none in other tissues except for the haemolymph where slight increases in the amounts of them occur in the mid-pupal stage, accounting to 1% of the content in the whole body. These increases in the haemolymph coincide with histolysis of the fat body. Lowered content of storage proteins in the fat body dissected from the animal in the mid-pupal stage even though the total contents of them are still high, can be caused by incomplete recovery of fat body cells which are in degenerating process (ref. Figs. 1 and 3). From these accumulation and distribution profiles of storage proteins, we speculate that SP-1 and SP-2 are consumed in the fat body, and not in other tissues.

As expected from the extraordinarily high content of storage proteins in the fat body in females and rather low content in males, accounting for 60 and 20% of the total fat body protein, respectively, of the newly pupated silkworm (Tojo et al., 1980), the amount of storage proteins in the whole body attains to about one fourth of the total protein in females, and only one twentieth in males, as reported here. In the females of E20 strain, SP-1 and SP-2 together amount to 19 mg per g wet weight, while in the males which lack SP-1, SP-2 amounts to 3 mg; females carry in the pupal stage excess amount of storage proteins, one of them as female limited form, by 16 mg per g over that in males. This corresponds with the fact that the total protein content in this stage is higher in females by 15–25 mg on g weight basis than in males (Tojo, 1971). We now understand that the female silkworms carry in the pupal stage additional protein mostly as storage proteins.

Another female-specific protein, vitellogenin, increases in the female silkworm pupa in a profile quite the reverse of those of storage proteins, and accumulates so as to represent 40% of the total protein in the newly ecdysed adult. Although vitellogenin and SP-1 have neither an immunological relationship nor any common subunit (Tojo et al., 1980; Izumi et al., 1980a), their amino acid compositions are closely similar except that the former contains more glutamate and serine, but lacks the exceptionally high methionine of the latter (Fig. 4). In calculation, omitting methionine and adding glutamate and serine convert SP-1 into vitellogenin. We have no direct evidence to support such a conversion, but some amino acid concentrations in the haemolymph of the silkworm change during pharate adult development as if to reflect it; glutamate and serine increase sharply in males, but remain constant in females, while cystathionine, which is a metabolic intermediate from methionine to cystine, remains low in males but increases prominently in females (Sumioka and Yoshitake, 1974). Cystathionine and another major sulphur amino acid of the haemolymph, lanthionine which is also synthesized from methionine, both being considerably higher in females than in males, are finally incorporated into the egg-shell protein (Inokuchi, 1972; Shinbo, 1978), the major sulphur amino acid of which is cystine (Kawasaki et al., 1971; Inokuchi, 1972; Sumioka and Yoshitake, 1974). These profiles of the changes in several compounds imply that SP-1 serves as a precursor of vitellogenin and surplus methionine from SP-1 is metabolized to cystine for chorion formation. During the pupal period of the silkworm, the decrease of SP-1 is about one half the amount of vitellogenin produced and theoretically together with SP-2 could provide a major part of the amino acids necessary to synthesize vitellogenin.

Insect vitellogenins are synthesized by the fat body, secreted immediately into the haemolymph, and sequestered into the yolk during oogenesis (Telfer, 1965;

Fig. 4. Comparisons of amino acid compositions of SP-1, SP-2, vitellogenin and calliphorin. Amino acid composition; SP-1 and SP-2 of *B. mori* from Tojo et al. (1980); vitellogenin of *B. mori* from Table 1; vitellogenin of *H. cecropia* from KUNKEL and PAN (1976); calliphorin of *C. erythrocephala* from MUNN et al. (1971). Relative contents of respective components are plotted on lines which divide equally a circle, based on their molar ratios. O, Centre of circle.

ENGELMANN, 1979). The present findings are consistent with this: vitellogenin is detectable only in the haemolymph in the early half of the pupal period and later increases in the ovary, while keeping a constant level in the haemolymph. So are the results from electrophoretic analysis (KAWAGUCHI and DOIRA, 1973), and tracer study on the synthesis of vitellogenin by the fat body (ONO et al., 1975). The developmental profile of vitellogenin in the whole silkworm during metamorphosis (ref. Fig. 1) suggests that the synthetic activity of vitellogenin by the fat body becomes elevated during pharate adult development, as in *H. cecropia* (PAN et al., 1969; PAN, 1971).

Vitellogenin isolated from *B. mori* is very similar to that from *H. cecropia* (KUNKEL and PAN, 1976), as illustrated in Fig. 4, and from *Philosamia cynthia* (CHINO et al., 1976). This suggests a homology of vitellogenin in these species of Lepidoptera.

In amino acid composition, SP-1 is also similar to the two storage proteins isolated from *Hyalophora cecropia* fat body, containing 12–13% tyrosine and phenylalanine together (Tojo et al., 1978), while SP-2 with 19% aromatic amino acids resembles to calliphorin, as compared in Fig. 4. Calliphorin and other related dipteran proteins are characterized by unusually high tyrosine and phenylalanine, and their use for cuticular protein formation has been inferred (MUNN et al., 1971; KINNEAR and THOMSON, 1975; THOMSON et al., 1976; WOLFE et al., 1977). Recently, a storage protein, manducin, has been isolated from *Manduca sexta* haemolymph (KRAMER et al., 1980), the amino acid composition of which is amazingly similar to that of SP-2. Manducin is retained in the haemolymph to occupy 66% of the total haemolymph protein at the larval-pupal ecdysis, which is rather similar to *H. cecropia* storage pro-

teins (Tojo, et al., 1978), but is different from SP-1 and SP-2 (Fig. 3).

Tracer studies are now in progress with the aim of elucidating the fate of two storage proteins in the silkworm.

ACKNOWLEDGEMENTS

We would like to thank Dr. G. R. WYATT of Queen's University for reading the manuscript. We are also indebted to Dr. O. YAMASHITA of Nagoya University for giving purified vitellogenin, to Dr. Y. NAGATA of Institute of Applied Microbiology, University of Tokyo for performing amino acid analysis, and to Drs. S. KIMURA and K. KIGUCHI of Sericultural Experiment Station for supplying silkworms. Thanks are also due to Drs. Y. MATSUMOTO and T. IKESHOJI of University of Tokyo for their advice and encouragement during this study.

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Note added in proof : Quantitative change of calliphorin during metamorphosis has been reported by SEKERIS and SCHELLER (1977) in *Calliphora vicina* in which calliphorin was shown to be incorporated into endocuticle during puparium formation by SCHELLER et al. (1980).

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