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Incorporation and Synthesis of Protein by the Ovaries of *Bombyx mori*

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Protein composition of the ovaries of ***Bombyx mori*** changes dramatically with the progress of pupal-adult development. Proteins which migrate moderately by disk electrophoresis increase first, faster moving proteins increase at later stages of development. Incorporation of homologous ^{14}C-FP rich fraction of haemolymph protein by the ovaries was shown to be most active during middle stages of pupal life. Transplantation of ovarian imaginal discs revealed different origin of egg proteins. Non-sex limited H-protein as well as female specific protein of the pupal haemolymph is transferred to the ovaries and accumulated in the egg cells. On the other hand, a protein which was referred to as "O" was shown to be synthesized endogenously by the ovaries.

INTRODUCTION

Sex-limited haemolymph proteins functioning in the formation of egg yolk have been demonstrated in various groups of insects since their discovery in ***Hyalophora cecropia*** by the elegant studies of Telfer (1954, 1960). Functionally similar female protein has also been found by electrophoretic techniques in the silkworm, ***Bombyx mori.*** In ***Bombyx*** female three sex-specific proteins are present, two of which are detected at only larval stage and were referred to as "Larval-female protein (FL)-1" and "FL-Z." Another is of the pupal haemolymph and was named "Pupal-female protein (FP)" (Doira, 1968). It was shown that FP is transferred from haemolymph to the ovaries where it comprises the protein fraction of egg yolk. This vitellogenic haemolymph protein, FP, is not synthesized by the ovaries but by other organs in the insect, as in the cases of vitellogenins in other species of Insecta (Doira and Kawaguchi, 1972; Kawaguchi and Doira, 1973).

Many investigators have obtained evidences, with electrophoresis and/or immunological techniques, which indicate that most of the haemolymph proteins are accumulated in the eggs. The situation is the same in ***B. mori.*** Whereas the vitellogenins are preferentially accumulated by the oocytes, other haemolymph proteins that enter the oocytes occur in higher concentrations in haemolymph than in yolk, It was shown that haemolymph proteins incorporated in the yolk were unaltered with regard to antigenic activities. Bell (1970) observed similarities in the diffusion rates and electrophoretic mobilities between the haemolymph and yolk antigens that indicate transfer of haemolymph proteins to

the yolk entails no gross changes in size or net charge.

Ovarian follicle cells of ***Anagasta kühniella*** at stages 5 and 6 produce proteins which are incorporated quickly into the oocyte. During stages 5 and 6 proteins are also incorporated from haemolymph (Cruickshank 1971).

In ***B. mori*** the incorporation of haemolymph protein into the ovaries was suggested to be most active in the middle stage of pupal life by the observation of changes of FP concentration during pupal development. The small egg mutant which produces no egg yolk served as a useful factor (Kawaguchi and Doira, 1973).

The purpose of this paper is to demonstrate the incorporation of haemolymph protein other than FP into ***Bombyx*** ovaries and to determine the stage of protein transport. Endogenous protein synthesis in developing ovaries was also examined.

MATERIALS AND METHODS

Bombyx stocks used in this experiments have been maintained under brother-sitser and/or cousin mating at this University (Chikushi, 1972). In the experiment of protein uptake by the ovaries through the injection of labelled haemolymph protein, r 03 strain that produces normal eggs was used. Mutant stocks for the egg character, d 41 and e ***26,*** were used in the transplantation experiment of the ovarian imaginal discs. Moths of the d 41 strain tend to lay kidney shaped eggs with normal black color under the control of a recessive ***ki*** gene. Eggs of the e 26 strain, on the other hand, have normal shape but colored red. Red egg character is manifested by a recessive ***re*** gene which locates on a chromosome different from ***ki.*** Transplantation of ovarian imaginal discs between those two strains was performed to distinguish the egg proteins which originated in pupal haemolymph from those which were endogenously synthesized during development of the ovaries. The procedures were carried out on larvae on the second day of the fifth instar. The body wall of the previously chilled female larva homozygous for ***re*** was wounded with a sharp razor at the position of right-spot of a paired star markings on the eighth segment, thereby right one of the paired imaginal discs of the ovaries was exposed. Each ovary was grasped with forceps and was cut free to remove. Ovarian imaginal discs were excised from female larva homozygous for ***ki*** and washed free of haemolymph with insect Ringer's solution. An undamaged disc from ***ki*** larva was implanted into the body cavity of previously ovariectomized (right side only) ***re*** larva soon after the operation. Animals of ***ki-ovary*** recipients surviving the procedure were allowed to go into further development. After the emergence, they were crossed with e 26 male moths homozygous for ***re.*** In some of the batches derived from the cross, both red-colored eggs of normal shape and black-colored eggs of kidney shape were mingled with. The former red eggs were produced in host's ovaries, while the latter black eggs were produced in implanted ***ki*** ovaries. Eggs of these two types laid by a single moth were used for protein analysis by disk electrophoresis.

Labelling of haemolymph protein

Female pupae of the r *03* strain were injected soon after the larval-pupal ecdysis with 1 μCi "C-algal protein hydrolyzate in 10 μl of distilled water. The animals were held at 25°C and bled 3 days after the injection. The clear haemolymph was gently added with one-third its volume of saturated ammonium sulfate solution and was centrifuged to remove globulin fraction, as it was already known that FP shows the solubility characteristic of an albumin. The supernatant was further added with ammonium sulfate to final **60** per cent saturation. The resulted precipitate was dissolved in 0.05 M phosphate buffer, pH 6.8, and was dialyzed overnight against the buffer at 4°C. More than 90 per cent of FP as well as some other counterpart for egg proteins presented in the pupal haemolymph were recovered in the final preparation. This sample was referred to as FP-rich fraction. Amount of label incorporated into FP-rich fraction was 110 cpm/mg protein.

Uptake of ^{14}C-FP rich fraction by the ovaries

Preparatory to injection the volume of ^{14}C-FP rich fraction was adjusted to **30,000** cpm/ml. Each 0.1 ml of the sample solution was injected into r 03 females on the lst, 3rd, 5th, 7th and 9th day after the larval-pupal ecdysis. The pupae were held at **25°C** for 6 hr to allow the incorporation of injected labelled protein into the ovaries. Duration of the pupal life of r **03** animals was 10 days after the ecdysis. After 6 hr incorporation of label the ovaries were dissected from the animals (2-40 insects per day studied), rinsed several times with the saline. Ovaries were homogenized in a cold teflon homogenizer containing a volume of 0.75 per cent saline equal in ml to twice the weight of samples in g. The resulting mixture was centrifuged at 900 g for 10 minutes to remove cell debris. Small part of the supernatant was subjected to protein determination by the Lowry method for measurement of total saline-soluble protein. Bovine serum albumin was used as a standard. The remaining part was subjected to perchloric acid precipitation (final 5 %). The precipitate was washed several times with 5 % PCA to remove free label if any and then collected on glass filter for radioassay. The radioactivity of the proteins was determined in a Beckman liquid scintillation counter.

Electrophoresis

In the experiment to examine endogenous protein synthesis and transfer of haemolymph protein by the ovaries, crude homogenate of egg with 0.75 % saline was directly applied to electrophoresis. In the experiment to show the changes of protein spectra during the development of ovaries, ovaries were homogenized in cold saline and centrifuged. The clear zone of the supernatant became the sample solution.

The original disk electrophoresis procedure of Ornstein and Davis (1962) was used with a slight modification to separate and analyze the proteins. The disk gels used in these experiments contained 6.5 % acrylamide instead of standard 7.5 %. Direction of the run was toward the anode. After the migration was complete, the gels were removed from the glass columns and fixed and

stained for protein in a 0.5% solution of coomasie blue in 10 % acetic acid for 1 hr, the excess stain was soaked out in 10 % acetic acid. Subsequently densitometry tracings of the protein patterns were obtained and quantitative evaluation was made by using an Ozmor Model AZ 82 densitometer.

RESULTS

Changes in protein composition of the ovaries during pupal development

The amount of saline soluble protein in a pair of ovaries of r 03 strain increased from approximately 0.15 mg in day-l pupa to 32.8 mg in day-9 (the day before emergence) pupa. The data presented in Fig. 1 show that from 1.9 to 8.2 per cent of the wet weight of the ovary consisted of soluble protein; the trend was to be toward a higher percentage of protein/weight as the ovary grew.

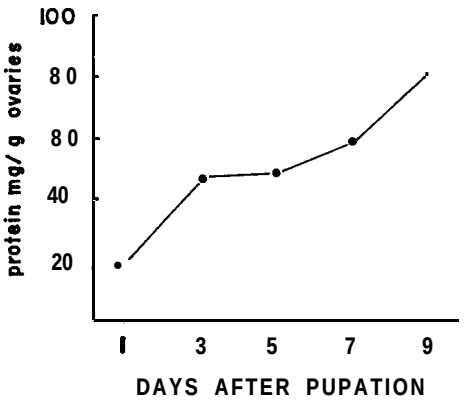

Fig. 1. Changes of protein concentration of ovaries during pupal development.

Qualitative changes in the protein (saline soluble) compositions of the ovaries during the pupal-adult transformation of r 03 strain are shown in Figs. 2 and 3.

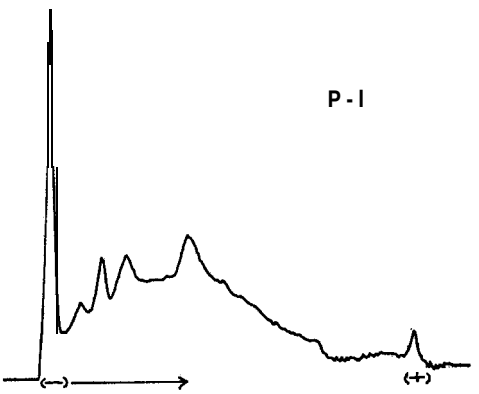

Fig. 2. Densitometric tracing of the ovarian protein of day-l pupa.

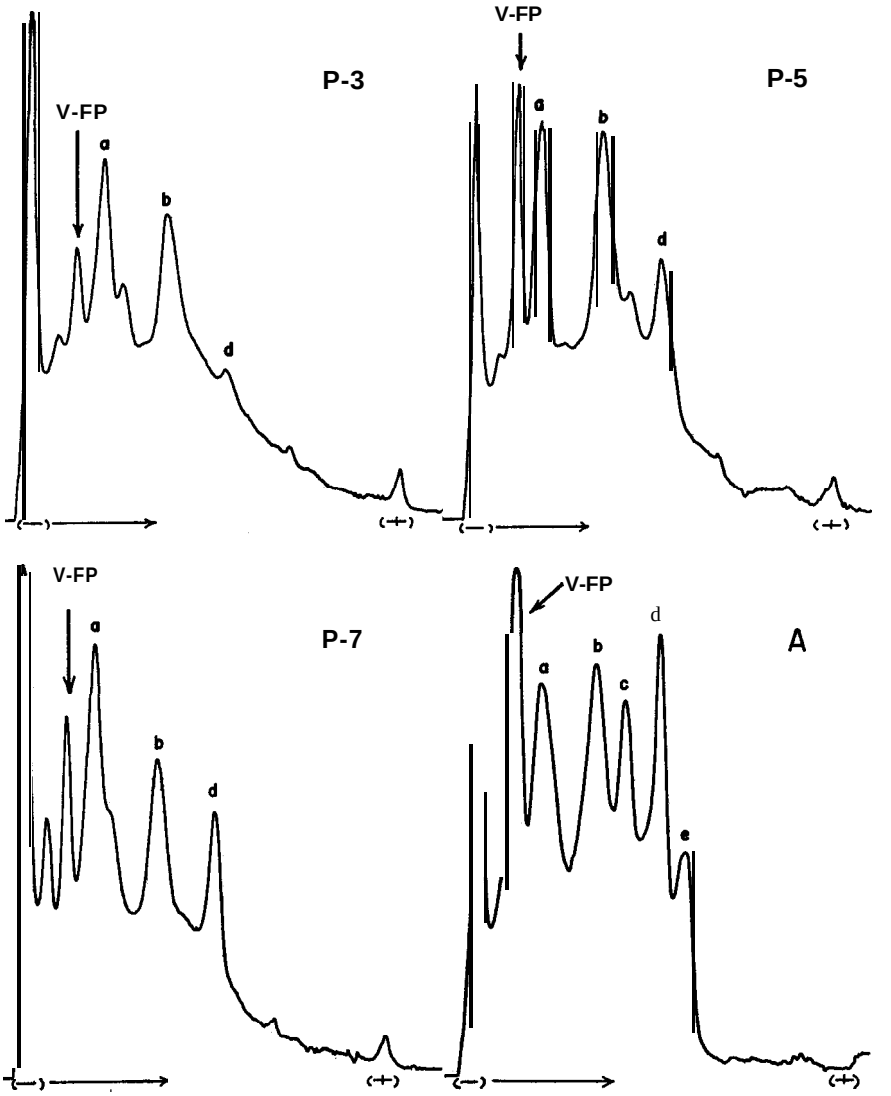

Fig. 3. Changes of protein compositions of ovaries during pupal development. Densitometric scanning after acrylamide gel disk electrophoresis. P-3 to P-7 : 3rd day to 7th day after larval-pupal ecdysis, A : adult, V-FP: vitelline-female protein.

Disk electrophoresis patterns were obtained at intervals of *2* days and a series of densitometry tracings was obtained from representative gels. The very young ovaries contained a large number of proteins, but it could not be established whether or not any of these were yolk proteins. Much of these material might be cellular and not associated with the yolk. In day-l pupa only five major area were resolved by disk electrophoresis (Fig. 2). A highly stained background in these area made the drawing of meaningful conclusions very

difficult. More clear separation of the protein bands was obtained in day-3 and older pupa (Fig. 3). Ovaries of day-9 pupa gave similar pattern to that of day-7 and omitted from illustration, instead protein pattern of mature egg obtained from virgin female was shown in the figure. That the relative proportions of the proteins change dramatically during pupal development was clearly demonstrated. This change in protein composition is characterized by the increasing tendency of V-FP and some other components with moderate mobilities, all of which were shown to be constituents of egg yolk and "FP-rich fraction" of the haemolymph. The increasing tendency of ovarian proteins can be divided into two phases. Before day-7, relatively slower moving bands increased remarkably, later increased faster moving bands.

Incorporation of labelled haemolymph protein by the ovaries

One of the criteria used to establish the existence of protein uptake by the ovaries was the transfer of injected homologous ^{14}C-proteins from haemolymph to the ovaries, In combination with the hope to identify the stage of development when haemolymph protein is transferred, the capacity of animals to incorporate labelled protein into the ovaries was tested as a function of the stage of metamorphosis (Fig. 4). The ability to incorporate labelled protein into the ovaries following injection of ^{14}C-FP rich fraction appeared on the first day after larval-pupal ecdysis; it remained at a relatively low level until day-3 and then increased rapidly, reaching a peak on the 5th day after the pupation, and finally fell off as the animals approached emergence.

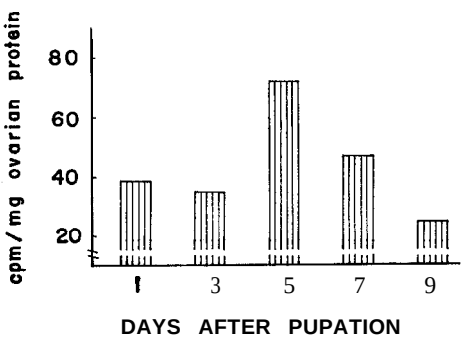

Fig. 4. Incorporation of injected ^{14}C-labelled FP rich fraction of homologous haemolymph protein into the ovaries.

Protein composition of eggs obtained through transplantation of the ovaries

By means of acrylamide gel disk electrophoresis, about ten protein bands can be detected in ***Bombyx eggs.*** The main band is V- FP which is transferred from haemolymph to the ovaries during pupal development. Protein patterns of representative gels are presented in Fig. 5. Gel-l represents protein pattern of e 26 eggs (re $^{+re}$), Gel-4 that of d 41 eggs ($^{+re}$; *ki*), respectively. Eggs of e 26 strain invariably had H-band which could not be detected in d 41 eggs. Eggs of d 41 strain, on the other hand, invariably had O-band of which mobility under the condition used was fairly slow. O-band, which was a minor component of

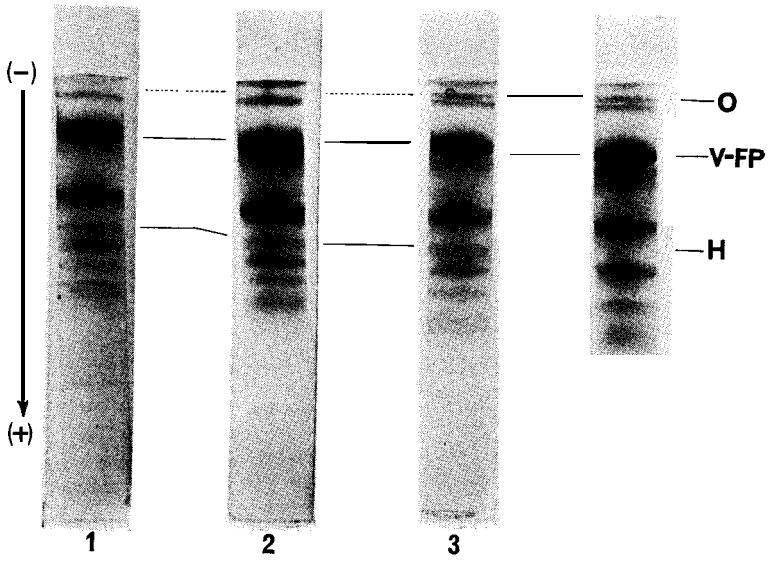

Fig. 5. Protein pattern of eggs obtained from ovary-implantation. (1) e 26-***red egg,*** untreated ; (2) ***red egg,*** produced in the ovary of e 26-host which received d 41 ovary, laid by the same moth as that of gel-3 ; (3) ***kidney egg,*** produced in d 41 ovary implanted into e 26 female ; (4) d 41-***kidney egg,*** untreated.

d 41 eggs, had no counterpart either in pupal haemolymph or in e 26 eggs, while H-band had its counterpart in e 26 pupal haemolymph but not in d 41 haemolymph.

Gel-2 and -3 represent protein patterns of eggs laid by an e 26 (re) female which had received a d 41 ***(ki)*** ovary and was crossed with e 26 male moth. Ked-colored eggs of normal shape which were produced in host's ovary showed protein pattern equal to non-treated e 26 eggs (Gel-Z). Black-colored eggs of kidney shape which were produced in implanted d 41-ovary also had O-protein band as in the case of non-treated d 41 eggs (Gel-3). Further, H-protein which could never be detected in eggs of d 41 strain made an appearance in those eggs produced in d 41 ovary implanted in e 26 female.

These results indicate that the O-protein is synthesized by implanted ovaries independently on host's genotype, while the H-protein is transferred to the implanted ovaries from host's haemolymph.

DISCUSSION

lnvestigations on the ***Bombyx*** silkworm have shown that in this insect a specific protein, FP, appears in female haemolymph soon after the pupation (Doira, 1968). The FP concentration increases rapidly until it makes up about 17 per cent of the total protein on the third day after ecdysis, then decreases in the middle and late pupal stage. In the absence of yolk formation, by ovariectomy or by mutation, FP is accumulated enormously in middle and later stages of pupal-adult development throughout (Doira and Kawaguchi, 1972 ;

Kawaguchi and Doira, 1973). Not only FP but also some other haemolymph proteins have their counterparts in the oocytes. These proteins are also accumulated in female's haemolymph in the absence of yolk formation. Measurements of total haemolymph protein level in ***Bombyx*** pupa thus have a diagnostic value in understanding the intricacies of the female reproductive cycle.

Incorporation of the injected ^{14}C-FP rich fraction into the ovaries provides an evidence that indicates exogenous origin of egg protein. Incorporation of label into the ovaries was most active on the fifth day after pupation. It was shown in previous reports cited above that the concentration of haemolymph protein was higher in female than in male prior to the fifth day after pupation, whereas in later stages of pupal-adult development the concentration was higher in male animals. Present results correspond well with this observation.

Amounts of label in the ovaries in later stages of development were unexpectedly high when we consider the fact that most of the oocytes were covered with layers of chorion. No conclusive explanation is possible at the moment to account for these observations. It might be associated with the simultaneous changes of ovarian protein spectra, i. e., increase of V-FP and moderately or slowly moving bands is shown before day-7 pupa while the increase of faster moving proteins, supposed to be of lower molecular weight, is characteristic to later stages. Though different groups of haemolymph protein may be incorporated at different stages of ovarian development, it is remained for further analysis.

Appearance of H-protein in kidney-shaped black eggs which were produced in d 41 ovary implanted into e 26 hemocoel proves that not only FP but also H-protein of the pupal haemolymph is transferred to the ovaries and accumulated in the yolk. H-protein of the pupal haemolymph is not sex-limited, and it decreases, during pupal development. to only traces in adult's haemolymph of both sexes.

In ***Bombyx*** silkworm it is now well established that the ovaries uptake proteins from haemolymph and deposit them in the yolk, some yolk proteins are synthesized also by the ovaries. Persistence of O-protein in the kidney-shaped black eggs produced in d 41 ovary which was developed and matured in e 26 female indicates that this protein is synthesized endogenously in the d 41 ovary itself, and also the absence in e 26 eggs is due to inherent genetic inabilities of the e 26 ovaries to do so. Repeated electrophoresis of d 41 haemolymph, and eggs and haemolymph of e 26 failed to yield protein band with a mobility similar to O-protein. O-band makes an appearance in only the eggs of d 41.

Present criteria to demonstrate the endogenous synthesis of egg protein is different in principle with those used in other insects. Reports on other insects are concerned mainly with the uptake of labelled amino acids, *in vivo* and ***in vitro,*** by follicle cells and subsequent transfer of radio-active grains to the oocytes. In ***Bombyx*** silkworm many inbred stocks with characteristic hereditary traits of the egg are available. More advanced insight as to synthesis and transport of egg protein may be derived from further studies.

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