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Changes in Haemolymph and Egg Protein by the Castration and Implantation of the Ovary in *Bombyx mori**

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During the pupal development of *Bombyx mori*, the haemolymph protein content decreases by about one-fourth. Slight change in the concentration was encountered in castrated females throughout pupal-adult development. Ovariectomy applied to females at larval stage does not influence the synthesis of pupal female protein (FP), which normally is found in the female blood of early pupa and then removed quickly from blood during the maturation of the ovaries. Castrated female is characterized by maintaining high FP concentration throughout the pupal life. Ovaries transferred to the hemocoel of males produced eggs but were unable to incorporate FP either into eggs they produced or into the blood of male ovary recipients. These evidences indicate that FP is secreted into the blood by some tissue other than the ovaries, and that it is subsequently transferred from blood to egg yolk. Another deficiency in the protein component of the eggs produced in male-implanted ovary was also demonstrated.

INTRODUCTION

The occurrence of sex-limited haemolymph proteins during progressive stages of metamorphosis of *Bombyx mori* has been reported previously (Kobara, 1967; Doira, 1968). In *Bombyx* silkworm, different from any other insects investigated so far, sex-limited haemolymph proteins take part in different form at the various stages in metamorphosis. In the fifth instar larvae female-specific proteins of the larval type are readily detected; they were separated first by thin layer electrophoresis on acrylamide gel and were referred to as Larval female protein (FL)-1 and -2. After pupation female proteins of the larval type disappear, instead, a faster moving protein is detectable in the haemolymph of female pupae. This female protein of the pupal stage was referred to as Pupal female protein (FP). That the FP plays the important role in vitellogenesis was also suggested in the course of these studies (Doira, 1968).

Telfer (1953, 1954, 1960) demonstrated the presence of a sex-specific and vitellogenic protein in the haemolymph of females of *Hyalophora cecropia*. Several authors have subsequently shown the occurrence of a female-specific and vitellogenic protein in animals belonging to various classes of Insecta (Laufer,

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1960; Hill, 1962; Coles, 1965; Engelmann and Penny, 1966; Thomas and Nation, 1966; de Loof and de Wild, 1970).

The changes, if any, of protein composition after ovariectomy and ovary-implantation into male animal must provide a clue to decide whether haemolymph protein is vitellogenic or not, and results of this study are presented in detail here.

MATERIALS AND METHODS

Bombyx stock used in this study was p 22 strain which has been maintained at Kyushu University since 1924 under brother-sister and/or cousin mating. It is a standardized pure line with normal traits in every respect and originated from a Japanese race "Yamato-nishiki".

Surgical procedures were utilized in these experiments designed to elucidate the function of FP. The procedures were carried out on animals on the second day of the fifth larval instar. Animals surviving the surgical procedure underwent complete development without any delay of growth.

Ovariectomy : The body wall of the previously chilled female larva was wounded with a sharp razor at the positions of a pair of star spots on the eighth segment, thereby the imaginal discs of the ovaries on each side were exposed. Each ovary was grasped with forceps and was cut free to remove.

Implantation of ovarian imaginal discs : Ovarian imaginal discs were excised from female larvae at the same age as in the case of ovariectomy, and washed free of haemolymph with insect Ringer's solution. Undamaged discs were implanted into the body cavity of previously chilled male larvae of the same age as donors.

Haemolymphs were obtained from pupae and adults at every 24 hours time interval for control animals and at every 48 hours for manipulated ones. The clear haemolymph was collected into a glass capillary after thrusting it into the intersegmental membrane of the abdomen. Matured eggs were collected by dissecting abdomen of newly emerged adults. The samples were used immediately after the collection or after storing at $-20°C$ until required for investigation. No centrifugation was attempted and instead the whole haemolymph or the whole aliquot of egg-homogenate was used.

Protein concentration of both the haemolymph and the egg was determined by the method of Lowry *et al.* (1951). Bovine serum albumin was used as a standard. Two different methods of acrylamide gel electrophoresis were applied; vertical disk electrophoresis according to Ornstein and Davis (1962) or horizontal thin layer electrophoresis according to Raymond and Weintraub (1959). After the migration was complete, the gels were stained for protein in a 1 % solution of amidoblack for 1 hr. Quantitative evaluation was made by using an Ozmor Model AZ 82 densitometer.

RESULTS

When haemolymph from the manipulated animals and from the control was compared visually, no change in yellowish color characteristic of the p 22

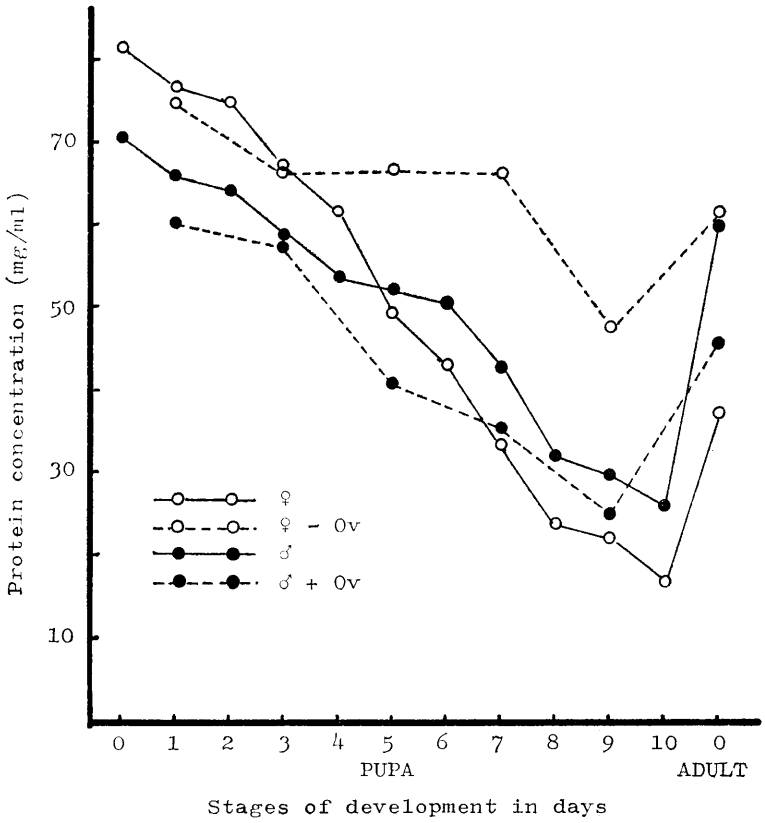

Fig. 1. Protein concentration of haemolymph after castration and implantation of the ovary during the progressive stages of pupal-adult's metamorphosis. ♀ : Control female; ♀-Ov: Ovariectomized female, ovarian imaginal discs on each side were excised on the day 2 of the 5th larval instar; ♂ : Control male; ♂+Ov: Ovary-implanted male, an ovarian imaginal disc which was excised from female larva on the day 2 of the 5th larval instar was implanted into male larva on the same day.

silkworm was observed throughout the life span. Fig. 1 shows the quantitative changes of blood protein during the pupal and adult stages. Each point represents average value from measurements of five times. A marked tendency of decrease in protein concentration was observed right from the stage of pupation, i. e., the maximum concentration of protein was observed on the day of pupation and it reached to minimum level on a day before emergence. In case of female control there was a rapid, almost linear, decrease up to 17mg/ml, whereas in male control the decrease did not extend beyond 26 mg/ml and also there appeared a "shoulder" during the day 4 and day 6 of pupal development. Further, there was a remarkable, rapid increase in the protein concentration from 17mg/ml to 37 mg/ml and 26 mg/ml to 60 mg/ml on emergence in female and male controls, respectively.

The protein concentration of ovariectomized females did not differ so much from that of control at least soon after the pupation, however, the protein

concentration remained constant from the day 3 to day 7 and then showed a decline (from 66 mg/ml to 47 mg/ml) on the day 9 followed by an immediate increase to 62 mg/ml on emergence. Contrary to the above, the protein concentration in ovary-implanted males showed a distinct tendency of decrease (from 60 mg/ml to 25 mg/ml) from the day 1 to day 9 with a "concave" on the day 5, and then increased to 46 mg/ml on the day of emergence.

These results indicate that presence or absence of the ovary is responsible for the amount of haemolymph protein in successive stages of pupal development. This suggests vitellogenic nature of the proteins in pupal haemolymph. It has been known that the yolk formation in this insect is most active in the middle stage of pupal life. Present results correspond well with the above.

When eggs from moths of male ovary recipients (hereafter referred to as Male-egg) and control female were compared visually, certain changes were very distinct. Eggs from the female moth were pale yellow in color. However, the yellowish color was scarcely recognizable in Male-egg. The shell of Male-egg was thinner than that of control. Preliminary experiment showed that the observed change in color is attributed to the amount of carotene and that also Male-egg is smaller than in control.

Eggs produced and matured in different environment of females or males were compared with each other as to the protein compositions. Table 1 presents weight and amount of protein in eggs both of the control and ovary-implanted male. Each value shown is the average of 10 replicates (confidence limit at the reliability of 95%). As shown in the table, Male-eggs were lighter in weight ($P<0.01$) and contained smaller amount of protein per egg ($P<0.001$). It is also clear that Male-eggs contained virtually smaller amount of protein per gram of eggs, though difference in between was not so clear ($0.10 > P > 0.05$).

Table 1. Protein content and weight of the egg produced in ovary-implanted male.

	Egg weight (γ/egg)	Protein content	
		γ/egg	mg/g of egg
Control	546±19	112±6	203.1±11.8
Male-implanted	471±22	89±5	188.8± 9.9

These results indicate that quantitative changes are caused in the egg protein by developing and maturing of the ovaries in peculiar environment as of male haemolymph.

Fig. 2 represents protein constitution of eggs produced in ovary-implanted male (gel B) and that of control eggs (gel A), respectively. In all about 13 fractions could be detected in the control. One of the major component showed a mobility not so different from that of FP of the pupal haemolymph. Identity between these two proteins of different origin has been proved with some properties as electrophoretic mobility under various pH and gel concentration, solubility and molecular weight. The distribution of "FP" in the yolk fraction of homogenate prepared from normal egg was also observed. The results will be presented elsewhere.

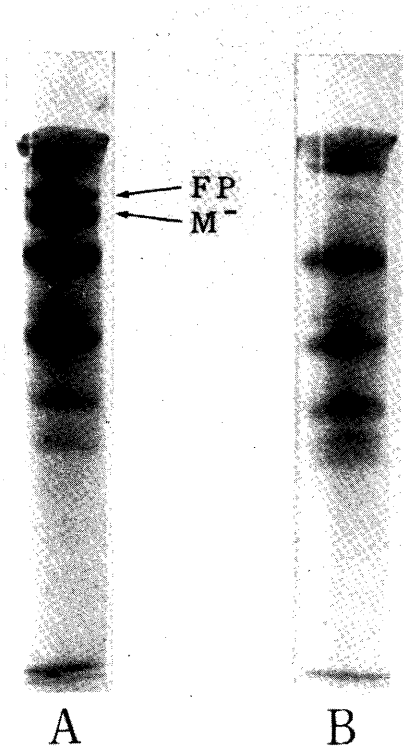

Fig. 2. Effect of ovary-implantation into male larva on the protein pattern of the egg (disk electrophoresis). (A): Pattern of a normal egg; (B): Pattern of an egg produced in male-implanted ovary. FP: Pupal female protein; M⁻: A protein which is readily detected in the control egg but not in the egg produced in ovary-implanted male.

Male-eggs had little amount of FP, and it was deduced from the fact that only trace amount of FP could be detected in the haemolymph of male animals throughout pupal life. Also, FP was not detected in pupal haemolymph of the male ovary recipients. Thus, the implanted ovaries, even though established well enough to produce eggs, are unable to synthesize FP in detectable amounts.

Furthermore, a component, showing a relatively slow mobility but faster than FP, which is invariably detected in control eggs occurred in traces in Male-eggs. This is certainly an unexpected result. The process resulting in the disappearance of this protein (hereafter tentatively referred to as M⁻ protein) in Male-egg is not clear at present.

Electrophoretic analysis showed that female pupa, which was ovariectomized at larval stage and then allowed to undergo pupal development, contained large amounts of FP in the haemolymph. On the third day of pupal development, FP reaches the maximum concentration of about 17 per cent of total protein in both control and castrated animals. In control female its concentration then falls quickly and it is present only in traces after emergence as was reported earlier (Doira, 1968). Meanwhile, ovariectomized females had high concentration

of FP throughout the later pupal development rather than undergoing the normal decrease. Fig. 3 shows the effect of ovariectomy on the composition of haemolymph protein of emerged adults. A larger amounts of FP as well as some others in the vicinity of it persisted into haemolymph of ovariectomized moth, while in control moth these proteins were not readily detected. These results indicate extra-ovarian origin of FP and its uptake by the ovary during yolk formation.

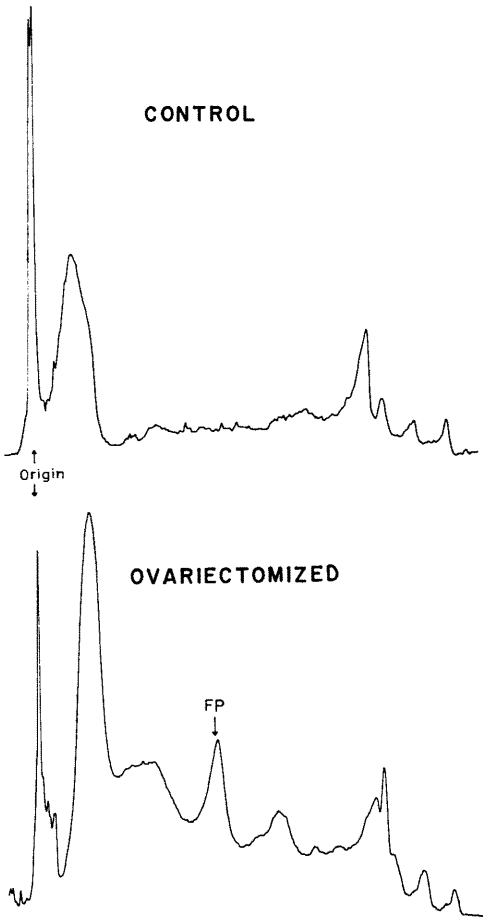

Fig. 3. Effect of ovariectomy on the haemolymph protein pattern of the adult. Densitometric scanning after the horizontal electrophoresis on the gel slab of acrylamide.

DISCUSSION

In ovariectomized females, the total protein concentration of haemolymph at the early pupal life closely approximates the value found in control of the same stage. Castration is characterized by maintaining the high protein concentration at the early stage throughout the pupal life, while in control females the protein concentration decreases quickly with the progress of pupal develop-

ment. Apparently the ovaries uptake the protein from pupal haemolymph.

Telfer (1953, 1954) demonstrated the occurrence of a female protein in the blood of adult females of *Hyalophora cecropia*. This sex-specific protein is selectively absorbed by the follicle cells of the ovaries and accumulated in the oocytes (Telfer, 1960). Subsequently, several authors showed the presence of a female specific and vitellogenic blood protein in various insects. In *Bombyx* female, Kobara (1967) and Doira (1968) independently observed the occurrence of sex-specific proteins.

During the transition from the spinning stage larva to the pupa of *B. mori* the female specific haemolymph protein switches from the larval type (FL 1 and -2) to the pupal one (FP). Present results provide evidences that FP is secreted into haemolymph by some tissue other than the ovaries, and that during the egg formation the ovaries transfer FP from the haemolymph and deposit it in the yolk. Thus, the amount of FP in the haemolymph, while normally decreasing during the egg formation, is caused to remain in large quantity throughout the later pupal-adult development when the animal is prevented from forming egg by ovariectomy. This indicates that some tissue other than the ovaries is capable of synthesizing FP at pupal stage becaus FP is readily detected in female blood first after pupation, neither in the blood nor in any other tissue of either sex at larval stage when the animals are ovariectomized. The ovaries themselves do not synthesize FP for the egg cells because they are unable to incorporate FP in the yolk when developed in an environment of male animal lacking this protein. Since only traces of FP is present in the haemolymph of male pupa, the large amount of this protein is presumed to be an expression of the female's potentialities for egg formation.

It is noteworthy that the synthesis of female-specific protein necessarily has no direct causal relationship with the development of the ovaries to form the eggs. These two processes are completely independent of each other, being controlled by different gene(s). Clear evidences in this line have already been obtained (Kawaguchi and Doira, unpublished). *Bombyx* females homozygous for the small egg mutant (*sm*, 3-41.8) produces sterile eggs which lack protein yolk sphere. It was shown that homozygous *sm* pupae synthesize FP and that a normal ovary implanted into a *sm* host will undergo normal vitellogenesis. The eggs of *sm*/*sm* female moths, on the other hand, contain only traces of yolk protein including FP, hence FP remains at a high concentration in the haemolymph of late pupae and adults throughout. This indicates that in the absence of the normal allele of *sm* silkworms are unable to incorporate FP into their oocytes. Attempts to find a mutant unable to synthesize FP are not successful yet.

As early as in 1925, Umeya reported an evidence which indicated that yolk pigments, carotenoids, of *B. mori* eggs are derived from the haemolymph. The yolk produced by the ovaries which had been transplanted between females of the normal and yellow-blood mutant (*Y*, 2-25.6) of silkworm invariably assumed the color of the host's blood. In the course of our experiment to purify FP, it was demonstrated that the blood pigments, carotenoids, are protein-bound, and that FP is one of the carotene-binding proteins. Such carotene-binding proteins are separated electrophoretically into five (or six) components (work in progress).

Therefore, we may safely infer that FP (and possibly M protein, too) of egg yolk is also carotene-bound, being incorporated from pupal haemolymph into the ovaries. Eggs produced in male-implanted ovary lack FP, which is a carrier protein of the pigments, and look whitish rather than normally pale-yellow. In the light of these evidences, it seems most likely that analogous mechanism of protein-bound active transport of the pigments is involved in Umeya's results.

The ovaries of cecropia silkworm implanted into diapausing male pupae were unable to synthesize female-specific "antigen 7" in either the blood or yolk (Telfer, 1954). This led him to consider that "the fact that the ovaries were able to produce eggs in the male suggests that their failure to synthesize antigen 7 was due to an inherent inability to do so rather than to an environmental deficiency". The situation is the same on principle in *Bombyx* as in cecropia because ovariectomized *Bombyx* females synthesize FP and accumulate it in late pupal and adult's haemolymph while ovaries implanted into growing male larvae were unable to synthesize FP in either the blood or yolk.

The apparent transfer of FP into normal ovaries but not into *sm* ovaries suggests that an active transport mechanism is involved there. However, the absence of M protein in the Male-egg might not be argued in favor of the active transport mechanism. Electrophoretic analyses of haemolymph proteins, both larval and pupal, using various constitutions of acrylamide gel, disk and slab, combined with buffers of various pH gave no evidence that M protein is also a female-specific haemolymph protein. Further studies on M protein are in progress.

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