家蚕卵の休眠過程に伴う卵特異蛋白質とビテリンの変動

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Quantitative and qualitative changes of polypeptides of egg-specific protein and vitellin depending on the diapause state of *Bombyx* eggs exposed to lower temperatures[1)]

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In order to define the changes in the protein polypeptides of *Bombyx* diapause eggs kept at 25°C, 5°C and 1°C, the protein patterns of these eggs were analysed on SDS-polyacrylamide gels. Two protein polypeptides with molecular weight of about 55 kDa derived from egg-specific protein (ESP), and 46 kDa from vitellin (Vtn) exhibited specific patterns depending on the diapause state of eggs subjected to prolonged exposure to low temperatures. The relative content of the 55 kDa ESP band always decreased at the end of the diapause, which that of the 46 kDa Vtn band showed an increase. When the eggs exposed to 5°C continuously were transferred to an environment at 25°C on day 140 or at the end of the diapause to induce embryogenesis, the relative content of the 55 kDa ESP band which was very low, increased abruptly with embryonic development. Accordingly, the 55 kDa and 46 kDa polypeptides may be associated with the embryonic diapause of *Bombyx* eggs.

The diapause in insects is characterized by a low metabolic rate and turnover of metabolites. In silkworm *Bombyx mori*, the embryonic diapause commonly induced by the active secretion of the subesophageal ganglion (Fukuda, 1952; Hasegawa, 1952; Yamashita, 1983) is associated with the metabolic pathways of glycogen and polyols (Chino, 1957, 1958; Yaginuma and Yamashita, 1978), even if the diapause states are controlled artificially by means of low temperature preservation of the eggs (Furusawa and Shikata, 1982; Furusawa *et al.*, 1982).

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To analyse the biochemical regulation of insect diapause and development, studies on the nucleic acids and protein metabolism may provide information on the physiology of the diapause. The RNA synthesis in *Bombyx* eggs during the diapause from the standpoint of gene expression (Kurata and Sakaguchi, 1977; Szyszko and Lassota, 1977) and calorimetric estimation of DNA content during the developmental stages (Kurata *et al.*, 1980) have been investigated. Furusawa *et al.* (1984, 1985) analysed the changes in RNA, DNA contents and RNA/DNA ratios to determine the physiological and biochemical status of the yolk and embryo in diapause and non-diapause eggs of the silkworm.

The role of the yolk proteins, especially the precursor of these proteins from the fat body, hemolymph and follicle cells has been studied

extensively (Wyatt and Pan, 1978; Engelmann, 1978; Borovsky and Van Handel, 1980). Among the yolk proteins of *Bombyx* eggs, the egg-specific protein (ESP) is considered to be the most important protein for embryogenesis (Irie and Yamashita, 1980, 1983; Zhu *et al.*, 1986). Recently, our studies (Indrasith, 1985; Indrasith *et al.*, 1987) on *Bombyx* egg-proteins during the embryogenesis have indicated that it may be possible to identify the developmental stages depending on the changes in the patterns of two specific protein bands, band-F (55 kDa) derived from ESP and band-H (46 kDa) from vitellin.

However, the role or function of the yolk proteins in the diapause states and the embryonic developmental stages of *Bombyx* eggs remains to be determined. In this report, the authors analysed the electrophoretic pattern of the silkworm egg proteins in which the diapause was controlled experimentally, to correlate the diapause states in *Bombyx* eggs with the changes in the levels of the protein polypeptides.

Materials and Methods

Experimental eggs: The eggs used for this investigation were obtained from a bivoltine strain (Asahi × Tokai) reared on an artificial diet (Takeda Co. Ltd., Japan). Incubation conditions were controlled to produce diapause eggs at a high temperature (25°C) and under a long day photoperiod (16L : 8D) regime during the embryonic period of the maternal generation.

Eggs were pooled within 3 hrs after the beginning of oviposition, and then maintained under the following temperature regimes; (1) continuous exposure to 25°C to retain the diapause state as controls, (2) continuous chillings at 5°C or 1°C staring 48 hrs after oviposition (3) continuous chilling at 5°C for 140 days after oviposition, and then at 25°C until hatching.

Sample preparation for electrophoresis : Fifty eggs at various diapause states were homogenized in a motar with 1 m*l* of 0.05 M Tris-HCl buffer (pH 8.2) containing 0.4 M NaCl and 1 mM PMSF, and the crude homogenate was dissolved in an equal volume of the buffer containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 25 mM Tris-HCl buffer (pH 6.8) and 0.06% bromophenol blue. The mixture was heated in boiling water for 3 min, and stored at −20°C until use.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE): The samples (30 μg protein) were electrophoresed on slab gels using a discontinuous buffer system (Laemmli, 1970), and at a constant potential of 30 V until the bromophenol blue reached at the bottom of the gel. The gels were fixed with 7% acetic acid for 30 min and stained with Coomassie brilliant blue G-250 according to the method of Blakesely and Boezi (1977).

Quantification of bands on SDS-PAGE: To estimate the relative content of the protein bands stained with Coomassie blue, the gels were scanned at 600 nm using a Shimadzu C-S 9000 chromatoscanner with a recorder (Shimadzu Co. Ltd., Japan) and the peak area were cut and weighted with a chemical balace. The relative content was expressed as cm^2/peak.

Hatching ratio: Samples of 100 eggs were removed at various times during cold preservation, and then incubated at 25°C for hatching. Hatched eggs were counted after 14 days to estimate the hatching ratio 14 days after the transfer of each sample to an environment at 25°C.

Results

Electrophoretic patterns of proteins from eggs exposed to low temperatures

Using the changes in protein bands as a biochemical index, it was possible to link the embryonic development with the physiological changes of the yolk cells in *Bombyx* eggs (Indrasith, 1985; Indrasith *et al.*, 1987). To further investigate the relation between the diapause states of the embryo and protein composition,

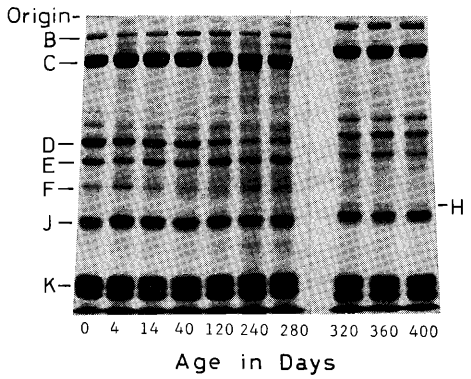

Fig. 1. 8% SDS-PGAE pattern of egg-proteins from a hybrid race (Asahi ×Tokai). The eggs were exposed to 25°C continuously. Each sample well contained 30 μg protein, and was stained with Coomassie brilliant blue G-250. The major bands of the egg proteins were determined as B; lipophorin subunit, C and J; vitellin heavy and light subunits, D, E and F; egg-specific protein subunits, H; 46 kDa polypeptide, K; 30 kDa proteins according to the results reported previously by Indrasith *et al.* (1987).

the proteins of eggs exposed to low temperatures were anlysed on SDS-PAGE.

When the eggs were exposed to 25°C continuously as control (Fig. 1), 7 major protein bands were observed. The staining intensities of the bands did not change except for the F- and H-bands until 400 days after oviposition.

Fig. 2a indicates the changes of the protein bands in the eggs kept at 25°C for 400 days after oviposition. The relative content of the F-band increased from the day of oviposition to about day 4, and remained constant until day 14, after which it declined. On day 120 the band could not be identified. In contrast the relative content of the H-band increased markedly from day 40. Diapause eggs which do not hatch normally when exposed to 25°C had a hatching percentage of about 50 on day 400 (Fig. 3).

When the eggs exposed to 25°C for about 48 hours after oviposition were transferred to an

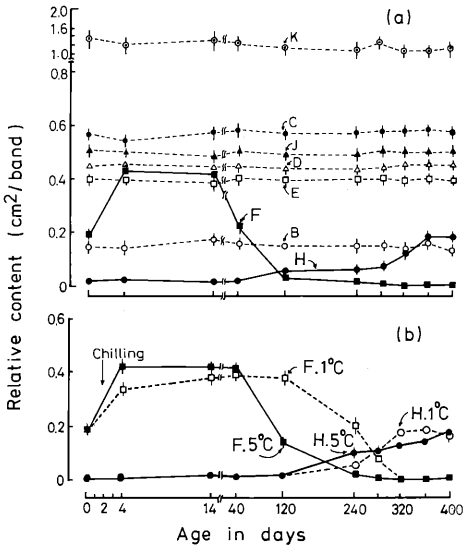

Fig. 2. Changes in the relative content of the protein bands separated by SDS-PAGE when the eggs were exposed to 25°C continuously (a), and some of these eggs were transferred to 5°C or 1°C 48 hrs after oviposition (b). Protein content is caluculated from the peak areas of each band (cm²/peak) after densitometric scanning. Each point represents the mean of three determinations with ± S. D. shown as vertical bars.

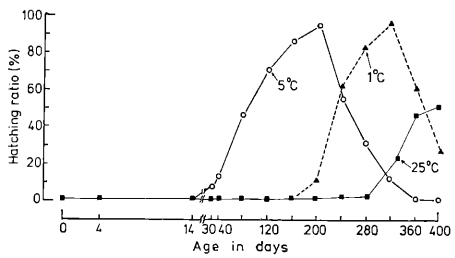

Fig. 3. Hatching ratios of eggs exposed to 25°C, 5°C and 1°C. The eggs were kept at 25°C for 48 hrs after oviposition and were then exposed continuously to three different temperatures. Egg samples were removed at various intervals, and incubated at 25°C for hatching. Hatched eggs were counted after 14 days to determine the hatching percentages.

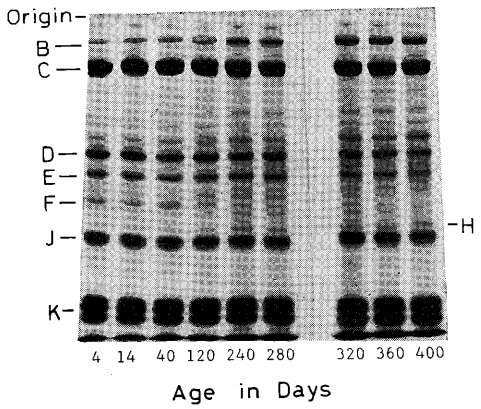

Fig. 4. 8% SDS-PAGE pattern of egg proteins from a hybrid race (Asahi × Tokai). The eggs were incubated at 25°C for 48 hrs after oviposition and then exposed to 5°C continuously. Each sample well contained 30 μg protein, and was stained with Coomassie brilliant blue G-250. Symbols are the same as in Fig. 1.

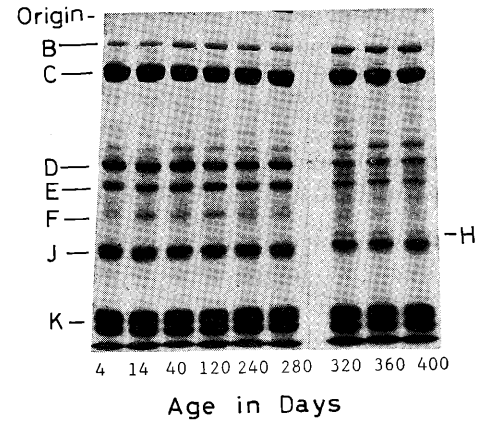

Fig. 5. 8% SDS-PAGE pattern of egg-protein from a hybrid silkworm race (Asahi × Tokai). The eggs were incubated at 25°C for 48 hrs after oviposition and then exposed to 1°C continuously. Each sample well contained 30 μg proteins and was stained with Coomassie brilliant blue G-250. Symbols are the same as in Fig. 1.

environment at 5°C or 1°C, the protein bands showed a different pattern in the staining intensities and the relative contents compared with the eggs exposed to 25°C continuously.

Fig. 4 shows the protein-band pattern of the eggs kept at 5°C. These eggs also had 7 prominent bands similar to those observed in the eggs maintained at 25°C as shown in Fig. 1. As indicated in Fig. 2b, the relative content of the F-band, however, remained constant during the diapause states from day 4 through 14, and decreased gradually at the end of the diapause (from day 40). On the 240 th day only traces were detected. These changes corresponded well with the changes in the staining intensity of the F-band. The marked decline in the F-band levels was observed at a later period in the eggs exposed to 5°C when compared to those preserved at 25°C (Fig. 2a). On the other hand, the level of the H-band which remained low up to day 40 (until the diapause termination), increased thereafter and reached the maximum value on day 400. As shown in Fig. 3, the rate of hatching of these eggs which was low but significant on day 40 was related closely to the increase in the relative content of the F-band during the same period.

Transfer of the diapause eggs after 48 hours of oviposition from an environment at 25°C to 1°C caused a decline in the rate of relative content of the F-band from 120 days (Fig. 2b) and this decline proceeded until day 300. At that time, the F-band had completely disappeared (Fig. 5). The decline in the relative content of the F-band was found to occur at a later period when compared to the eggs exposed to 25°C and 5°C continuously. Correspondingly, the relative content of the H-band began to increase markedly 120 days after the oviposition and the marked increase in the hatching ratio (Fig. 3) of these eggs corresponded well with the increase in the relative content of the H-band.

Based on these results, it appears that the responses of the F-band and H-bands to low temperatures such as 5°C and 1°C were quiet different. The relative content of the F-band

always decreased when the diapause ended, whereas that of the H-band increased during the same period.

Changes in the patterns of the F-and H-bands during embryogenesis

Hatching ratio of the eggs exposed to 5°C (Fig. 3) shows that the induction of the diapause termination began from about 30 days after oviposition, and the relative content of the H-band increased correspondingly. At 100 days after oviposition the hatchability of these eggs decreased suddenly, suggesting that the eggs need at least 200 days to develop to the diapause embryo when they were continuously exposed to 5°C after the first 48 hrs of exposure to 25°C.

To analyse the electrophoretic patterns of these diapause eggs, some of these eggs exposed continuosly to 5°C were transferred to an environment at 25°C on day 140 to induce embryogenesis. The electrophoretic pattern is shown in Fig. 6. The major 7 bands were observed from day 140 through 146, but the staining intensities of the F- and H-bands increased with the embryogenesis. On day 147 (7 days after the transfer to 25°C), the staining intensity of most bands became weaker, and 3 new bands (G, H and I) with high staining intensities appeared. These bands also became invisible one day before hatching.

As shown in Fig. 7, the relative content of the F-band, which showed the lowest value on day 140 increased gradually up to the 142 nd day and the level remained almost constant until day 145, after which it decresed markedly. On the other hand, the relative content of the H-band

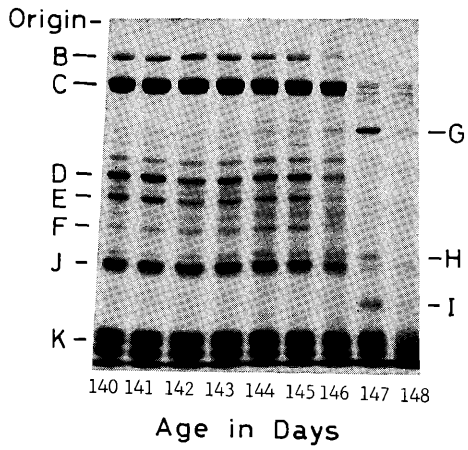

Fig. 6. 8% SDS-PAGE pattern of egg-proteins from a hybrid silkworm race (Asahi × Tokai). The eggs were exposed to 5°C and then transferred to an environment at 25°C on day 140. Each sample well contained 30 μg protein, and was stained with Coomassie brilliant blue G-250. Symbols except G and I as the same as in Fig. 1. G and I designate the 96 kDa protein from vitellin and 36 kDa protein from egg-specific protein, respectively according to the results of a previous report (Indrasith *et al.*, 1987).

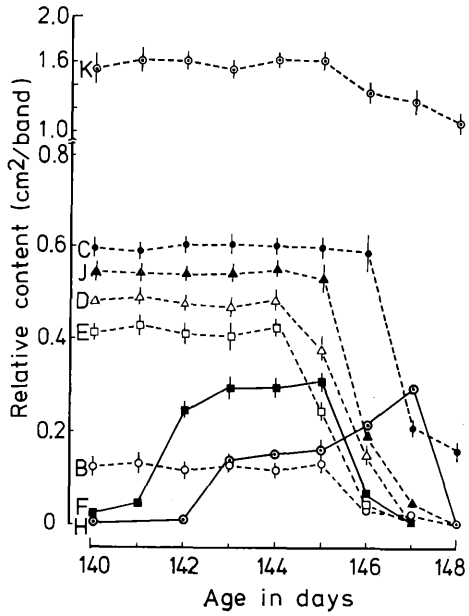

Fig. 7. Changes in the relative content of protein bands separated by 8% SDS-PAGE when the eggs exposed to 5°C were transferred to an environment of 25°C on day 140. Protein content is calculated from peak areas of each bands as (cm^2/band) after densitometric scanning. Each point represents the mean of three determinations with ±S.D. shown as vertical bars.

increased rapidly on day 142 and reached the highest level on day 147. On day 148 this band was almost invisible. The results of both the electrophoretic patterns and the relative content of the bands in these eggs resembled those recorded in the non-diapause eggs of N_4 and Daizo (Indrasith *et al.*, 1987). The disappearance of the F-band during the prolonged chilling at 5°C and its re-appearance during the induced embryogenesis at 25°C suggest that the protein of the F-band may be accumulated in the embryo at early stages of the development for use during the embryonic differentiation.

Discussion

The proteins of *Bombyx* eggs exposed to low temperatures such as 25°C, 5°C and 1°C were analysed on SDS-polyacrylamide gels to determine their biochemical characteristics during cold treatment.

In the eggs kept at 25°C as controls, the relative content of the F-band increased from day 0 to 4, and remained almost constant untill day 14, after which it began to decrease. On the other hand, the relative content of the H-band increased gradually from day 40. Diapause eggs do not hatch normally when exposed to 25°C, but the eggs used in this experiment showed a hatching percentage of about 50 on day 400 (Fig. 3), because these eggs were derived from moths which had been reared on an artificial diet. Therefore, the eggs may have lost their diapause states or it became weakened due to reared on artificial diet and photoperiodic changes. Thus the diapause developmental stage may have progressed during the period of exposure to 25 °C.

In the eggs exposed to 5°C, the level of the H-band increase after the diapause termination (day 40), and that of the F-band decreased from this period. The same results were obtained in eggs exposed to 1°C, where the H-band level increased from day 120, and the F-band level decreased from the same day (Figs. 4 and 5). These results indicate that at a temperature of 25°C the accumulation of the H-band protein occurs more rapidly when compared to the temperatures of 5°C and 1°C. On the other hand, the F-band level decreased during this period. These results probably indicate that the diapause development and its termination can be monitored from the changes in the levels of the F-band and H-bands, which corresponded to the increase in the DNA content (Furusawa *et al.*, 1985; Indrasith, 1985), suggesting that the protein of the F-band may be utilized for cell division at the end of the diapause.

When the eggs exposed to 5°C for 140 days were transferred to an environment at 25°C, the level of the F-band which was very low on this day increased again and the changes were similar to those observed in the non-diapause eggs reported already by Indrasith *et al.* (1987). This may be due to the fact that the protein of the F-band which was detected in the yolk in larger amount than in the embryo was degraded and then synthesized during cold acclimatization and normal development.

The major 7 bands (B, C, D. E, F, J and H) shown in Figs. 1, 4 and 5' and the two bands (G and I) in Fig. 6, corresponded to those on SDS-PAGE detected by Indrasith *et al.* (1987). Therefore, it is suggested that the B band corresponded to the lipophorin heavy subunit (200 kDa), C-and J-bands to vitellin heavy (178kDa) and light subunits (43 kDa), respectively, D-, E- and F-bands to the egg-specific protein subunits (72 kDa, 64 kDa and 55 kDa), G- and H-bands to the polypeptides (96 kDa and 46 kDa) derived from vitellin, I-band to the polypeptide (36kDa) derived from the egg-specific protein, and the K-band to the 30 kDa proteins.

Accordingly, it is concluded that the protein polypeptide of the F-band (55 kDa) derived from the egg-specific protein, and that of the H-band (46 kDa) derived from vitellin were closely re-

lated to the diapause termination and embryogenesis of *Bombyx* eggs, respectively, also suggesting that the two proteins may play a part in the biochemical adaptation to low temperatures such as 5°C and 1°C.

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古沢寿治・レスリー S. イントラシット：家蚕卵の休眠過程に伴う卵特異蛋白質とビテリンの変動

家蚕卵の休眠状態と卵特異蛋白質 (ESP) およびビテリン (Vtn) との関連を追求するため，産卵48時間後に5°Cと1°Cに保護し，休眠過程に伴う蛋白質パターンの変動を SDS-ポリアクリルアミドゲル電気泳動法によって調べた。その結果，主として7本のバンドがみられ，このうち，休眠覚醒に伴い顕著に変動したバンドは ESP 由来蛋白質 (分子量55kDa) と Vtn 由来蛋白質 (分子量46kDa) であった。すなわち，5°C保護卵では産卵40日後から，また，1°C 保護卵では産卵160日後から休眠覚醒が始まった。いずれの保護卵でも覚醒期に対応して，産卵直後から増加した55kDa ESP が減少を始め，46kDa Vtn が増加した。さらに，5°C 保護卵を産卵140日後に25°C へ移したところ，55kDa ESP が胚発育に伴って再び増加した。これらの結果から，55kDa ESP と 46kDa Vtn の変動は，休眠状態と密接に関連していることが判明した。