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The Fate of Vitellogenin and Site of Protein Degradation during Oosorption in the Brown-winged Green Bug, Plautia crossota stali 【Research report】

褐翅綠椿象卵再吸收作用中卵黃原蛋白的命運及蛋白質崩解地點【研究報告】

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Abstract

When oocyte resorption or oosorption was induced by food deprivation in the brown-winged green bug, Plautia crossota stali, the protein and vitellogenin contents in the ovaries decreased. In the present study, observations were made to determine if the yolk protein, vitellin, was released from the ovaries into the hemolymph without degradation during oosorption. Western blotting using anti-vitellogenin serum showed two major and several minor bands in hemolymph samples from females with developing ovaries. Ovary extracts showed a more-complex set of positive bands in the same analysis. Vitellin, therefore, seemed to be formed after processing of vitellogenin in the oocytes. Western blotting failed to detect either oosorption- or ovary-specific peptide fragments in hemolymph samples collected from starved, oosorption-induced females. On the other hand, an immunohistochemical technique showed that anti-vitellogenin-positive materials were taken up by follicle cells in those females. Film in situ zymography demonstrated a high level of protease activity in follicle cells from starved females, whereas only a low level of activity was detected in females with developing ovaries. These results suggest that once oosorption is induced vitellogenin/vitellin in the oocytes is taken up and rapidly degraded by follicle cells, and the resulting amino acids or small peptides, which are too small to be recognized by anti-vitellogenin serum, are probably released into the hemolymph.

摘要

當剝奪食物的供給，造成褐翅綠椿象卵細胞再吸收作用時，卵巢內的蛋白質及卵黃原蛋白會減少。在這個研究中，觀察卵黃蛋白是否會被完整地從卵巢釋放至血淋巴中，而不會在卵再吸收中被分解。西方墨點法利用抗卵黃原蛋白血清從卵巢正在發育雌蟲的血淋巴標本中顯現二條主要和許多不重要條斑，而卵巢萃取物在同樣的分析中反而呈現一套更複雜的條斑。因此卵黃蛋白很可能在卵細胞進行卵黃原蛋白累積之後才形成，西方墨點法無法從飢餓引起卵再吸收作用的雌蟲血淋巴樣本中偵測到卵再吸收或卵巢專屬胜肽片斷。另外，免疫組織染色技術發現，在此類雌蟲中，卵黃蛋白物質會被濾泡細胞吸收。利用原位酵素圖譜證明，在飢餓雌蟲的濾泡細胞內有高程度的蛋白酶活性，而在卵巢正在發育的雌蟲中，只偵測到低程度的活性。這些結果建議一旦卵再吸收作用被誘導出來，在卵細胞的卵黃原蛋白 / 卵黃蛋白會被濾泡細胞吸收及快速分解，結果造成胺基酸或小的胜肽可能被釋放於血淋巴中，由於太小以致於無法被抗卵黃原蛋白血清辨認。

Key words: ovary, yolk protein, starvation, proteolysis, Pentatomidae
關鍵詞: 卵巢、卵黃蛋白、飢餓作用、蛋白質分解、椿象科

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The Fate of Vitellogenin and Site of Protein Degradation during Oosorption in the Brown-winged Green Bug, *Plautia crossota stali*

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ABSTRACT

When oocyte resorption or oosorption was induced by food deprivation in the brown-winged green bug, *Plautia crossota stali,* the protein and vitellogenin contents in the ovaries decreased. In the present study, observations were made to determine if the yolk protein, vitellin, was released from the ovaries into the hemolymph without degradation during oosorption. Western blotting using anti-vitellogenin serum showed two major and several minor bands in hemolymph samples from females with developing ovaries. Ovary extracts showed a more-complex set of positive bands in the same analysis. Vitellin, therefore, seemed to be formed after processing of vitellogenin in the oocytes. Western blotting failed to detect either oosorption- or ovary-specific peptide fragments in hemolymph samples collected from starved, oosorption-induced females. On the other hand, an immunohistochemical technique showed that anti-vitellogenin-positive materials were taken up by follicle cells in those females. Film *in situ* zymography demonstrated a high level of protease activity in follicle cells from starved females, whereas only a low level of activity was detected in females with developing ovaries. These results suggest that once oosorption is induced vitellogenin/vitellin in the oocytes is taken up and rapidly degraded by follicle cells, and the resulting amino acids or small peptides, which are too small to be recognized by anti-vitellogenin serum, are probably released into the hemolymph.

Key words: ovary, yolk protein, starvation, proteolysis, Pentatomidae

Introduction

Oosorption is a phenomenon in which developing oocytes are resorbed in the ovaries in response to internal and/or environmental factors, and is a specific strategy for reproduction that conserves resources and insures reproductive success (Bell and Bohm, 1975). Therefore, this phenomenon represents an adaptive response to adverse conditions for reproduction or survival rather than an abnormality. Various environmental, behavioral, and internal factors are involved in the

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induction of oosorption in many insects (Bell and Bohm, 1975). Although this phenomenon is well documented in various insects, neither biochemical or morphological changes associated with oosorption nor its regulatory mechanisms are fully understood.

The brown-winged green bug, *Plautia crossota stali,* is known as an important pest of fruit trees in Japan (Shiga, 1980). It also occurs in China and on the Korean peninsula. During a study to examine the physiological aspects of ovarian development in this species, I found a small number of females with ovaries undergoing oosorption even under reproduction-promoting conditions. The present study was thus initiated to elucidate the physiological mechanism of oosorption in *P. c. stali*. The accumulation of a huge amount of yolk is the most outstanding feature in egg production (Raikhel and Dhadialla, 1992). It is during this process that oosorption is induced by environmental factors such as starvation in this species (Kotaki, 2003). One of the major questions addressed in this study is: What happens to the yolk protein, vitellogenin (Vg) or vitellin (Vn), already taken up by the oocytes after the onset of oosorption? This paper summarizes the fate of vitellogenin in the ovaries (Kotaki, 2003) and the function of the follicle cells as the site of protein degradation during oosorption (Kotaki, 2005).

Materials and Methods

Insects

A stock culture of *P. c. stali* was established from adults collected in the field at Tsukuba and Mitsukaido, Ibaraki, Japan and kept for more than 10 generations under long-day conditions (LD 16:8 h) at 25°C in the laboratory. The rearing method was described elsewhere (Kotaki, 1996). Briefly, insects were maintained on dry soybeans and raw peanuts along with water supplemented with sodium L-ascorbate (0.05%) and L-cycteine (0.025%). A group of 14 nymphs was reared in a plastic Petri dish (9 cm in diameter and 2 cm in height) under long-day conditions at 25°C. Upon adult emergence, two female-male pairs were confined in each Petri dish under the same conditions. To induce oosorption, females were deprived of food 4 days after adult emergence (Kotaki, 2003). Hemolymph samples were collected with a glass capillary tube from a puncture at the neck membrane, and the ovaries were taken out of females under a dissecting microscope after experimental females were immobilized on ice. These samples were kept at -20°C until used.

Measurement of vitellogenin, electrophoresis, and Western blotting

Ovary samples were homogenized in extraction buffer (0.1 M Tris-HCl (pH 8.5) containing 0.25 M NaCl, 0.1% Triton-X 100, and a mixture of protease inhibitors for mammlian cell and tissue extracts (Wako, Osaka, Japan)), and hemolymph samples were diluted with the same buffer. The supernatant of the ovary homogenate was saved as the ovary extracts after centrifugation at 2000 *g*. Protein contents in the samples were determined using the Advanced Protein Assay reagent (Cytoskeleton, Colorado, USA). Vg contents were measured using a rocket immunoelectrophoretic (RIE) technique as outlined by Laurell (1966) with some modifications along with anti-Vg serum developed in a rabbit (Kotaki, 2003). The hemolymph samples and ovary extracts were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide-gel electrophoresis (PAGE) using a gradient gel of 5-20%. For Western blotting, protein samples resolved by SDS-PAGE were transferred to a polyvinilydendifloride membrane. To detect Vg/Vn, the anti-Vg serum and a Vectastain ABC Rabbit IgG kit (Vector Laboratories, California, USA) were used. Details of these methods were

described elsewhere (Kotaki, 2003).

Histological technique and film *in situ* zymography

To prepare histological sections, ovaries were fixed in 4% formalin in phosphate-buffered saline, and embedded in a glycol methacrylate (GMA) resin, Historesin-Plus (Leica, Heidelberg, Germany) after dehydration in an alcohol series. Sections were cut using a rotary microtome at 3-4 μm thick and stained with toluidine blue. To localize vitellogenin in the sections, the anti-Vg serum and the Vectastain ABC Rabbit IgG kit were used. Film *in situ* zymography (FIZ) was adopted to visualize the protease activity in frozen sections. Briefly, ovaries which were freshly excised were frozen in a medium, Tissue-Tech OCT Compound (Sakura Finetechnical, Tokyo, Japan) and cut into 4-μm-thick sections using a cryostat. These sections were then transferred to a gelatin-coated plastic film, MMP *in situ* Zymo-Film (Wako), and kept for about 6 h in a moist box at room temperature. The film was treated with a biebrich scarlet staining solution (Wako) after being air-dried. With this method, proteinase activity in the sections was visualized as a white area on a red background.

Results

Morphological and histological observations

The ovaries of fed females kept under long-day conditions at 25°C developed rapidly after adult emergence (Fig. 1A). On day 2 of adult life, the ovarian follicles had already differentiated. During the following 5 days, the size of the terminal oocytes greatly increased. The first female with mature eggs in the oviduct was observed on day 6. When females were deprived of food on day 4, their terminal oocytes remained small compared with those of fed females under the same conditions (Fig. 1A), and on the 4th day of starvation, all females examined had ovaries undergoing oosorption (Fig. 1B).

Protein and Vg contents in the ovaries in fed females remained at low levels during the first 3 days of adult life (Fig. 1C, D). On day 4, the protein and Vg contents began dramatically increasing and reached a peak value on day 14. In starved females, the contents slightly increased on days 5 and 6 and then decreased to a level similar to that at the beginning of starvation (day 4).

The terminal oocytes in the vitellogenic stage in fed females were mainly filled with yolk granules and lipid droplets. A monolayer of follicle cells surrounding the oocytes was clearly seen in those females (Fig. 2A). In the lateral and posterior parts of the follicles, spaces between the follicle cells, through which the developing oocytes are known to take up Vg from the hemolymph (Raikhel and Dhadialla, 1992), could be seen (Figs. 2A, 3A). After food deprivation, the first change observed in the ovaries was the disappearance of the spaces between follicle cells (Fig. 2B). At this stage, however, yolk granules had not yet broken. Following 2-3 days of starvation, yolk granules began to degenerate, the follicle cell layer increased in thickness, and a large number of vacuoles were found in follicle cells (Figs. 2C, 3B). At a later stage of oosorption, the contents in the oocytes decreased in volume. As a result, the space between this layer and the ovarian sheath could clearly be seen (Fig. 2D), the ovarian sheath became thicker, and the follicle cell layer underwent folding in a complex way (Fig. 3C). Yolk granules were no longer observed at this stage. As oosorption proceeded further, the nuclei of some follicle cells appeared to have broken into several fragments and become condensed.

The immunohistochemical technique revealed that only yolk granules were positive against the anti-Vg serum in

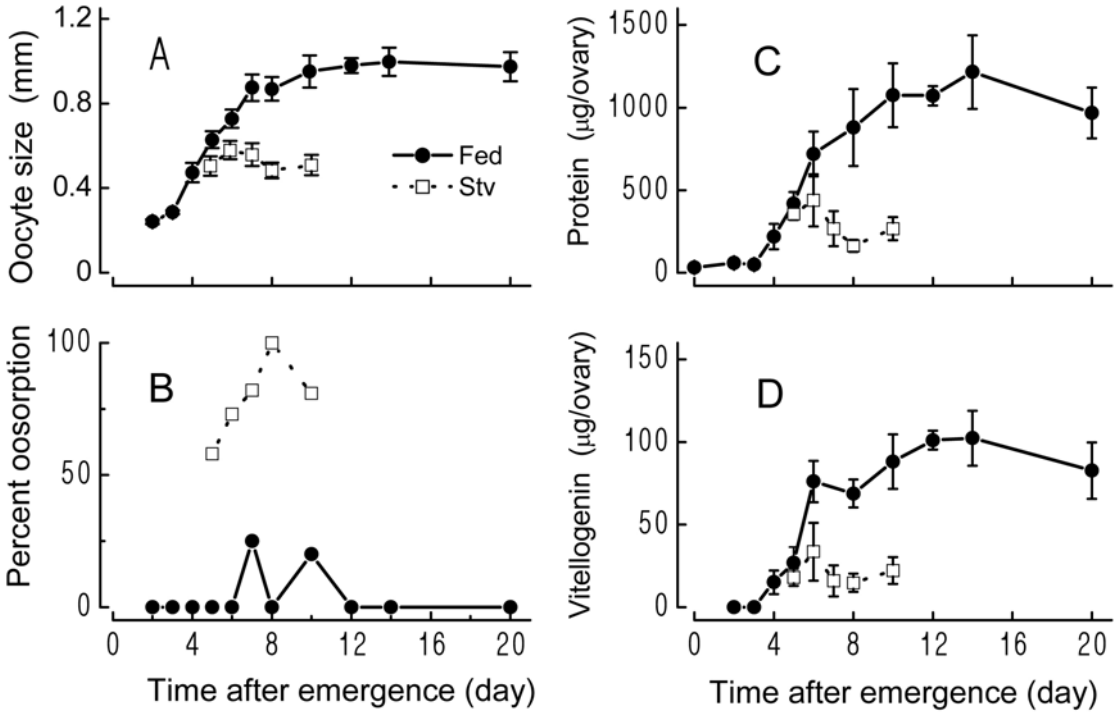

Fig. 1. Oocyte size (A), the occurrence of oosorption (B), and total protein (C) and vitellogenin contents in the ovaries (D) of *Plautia crossota stali* females starved from day 4 (open squares) and fed females (closed circles). A, The size of the terminal oocytes was determined in adult females. Symbols with vertical bars indicate the average ± SEM. B, The percentage of females undergoing oosorption in each experimental group. C and D, Total protein and vitellogenin contents were determined. Symbols with vertical bars indicate the average ± SEM. Each datum point in A-C was based on at least 10 insects, and at least five in D. (Modified from Kotaki (2003) with permission of Elsevier Science, Amsterdam, Netherlands)

developing oocytes (Fig. 3A). The follicle cells at this stage contained no positive material against the antiserum. When oosorption was induced and the yolk granules degenerated, the oocyte contents other than lipid droplets were evenly stained with the antiserum. In follicle cells, vesicles in the vacuoles reacting with the antiserum were frequently observed (Fig. 3B). At a later stage of oosorption, no staining of either follicle cells or oocyte contents was observed (Fig. 3C).

Western blotting of hemolymph and ovary extracts

To follow the fate of Vg/Vn during oosorption, ovary extracts and hemolymph collected from starved females were subjected to Western blotting using the anti-Vg serum. In hemolymph samples taken from reproductively active, fed females, a set of bands was recognized by the antiserum (the lane with the label, Fed, in Fig. 4A). This set consisted of two remarkable bands, whose masses were estimated to be about 140 and 50 kDa, respectively, and several minor ones. A different, more-complex set of bands positive against anti-Vg serum was observed in ovary extracts from fed females (the lane with the label, Fed, in Fig. 4B). In

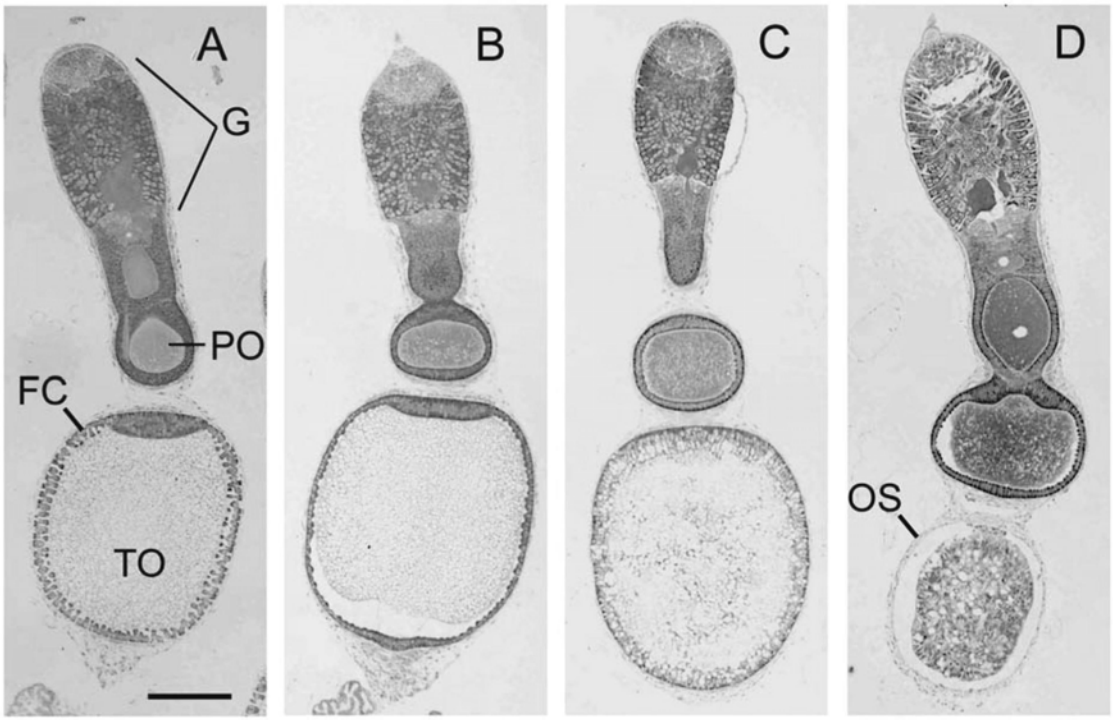

Fig. 2. Ovarioles excised from fed (A) and starved females (B-D) of *Plautia crossota stali.* Note that intercellular spaces between follicle cells (FC) surrounding the terminal oocyte (TO) were observed in the ovariole undergoing normal vitellogenesis in A; the intercellular space had disappeared in the ovariole taken from females starved for 2 days in B; thickened follicle cells with many vacuoles in the degenerating follicles in females starved for 4 days in C; and the follicle in the late stage of oosorption taken from a female starved for 6 days in D contained little oocyte contents. PO, penultimate oocyte; G, germarium. The GMA resin sections were 4 μm thick and stained with toluidine blue. Scale bar: 0.2 mm. (Modified from Kotaki (2005) with permission of Balaban, Rehovot, Israel)

ovary extracts from starved females, similar sets of positive bands were also observed with additional bands of about 37 and 23 kDa appeared after 3-4 days of starvation. The latter bands were not observed in the samples collected after 6 days of starvation.

Qualitatively the same set of positive bands was observed in hemolymph samples collected from fed females and females starved for up to 6 days, and no positive band specific to either starved females or ovary extracts was detected (Fig. 4A).

Film *in situ* zymography (FIZ)

When frozen sections of ovaries undergoing oosorption were subjected to FIZ, a ring-shaped white area, corresponding to the follicle cell layer, was clearly revealed (Fig. 5B). This white area indicated a high level of protease activity compared with the other areas. In control ovaries from fed females, on the other hand, no conspicuous protease activity was found (Fig. 5A). During a prolonged, overnight incubation of the control sections, however, the gelatin under the

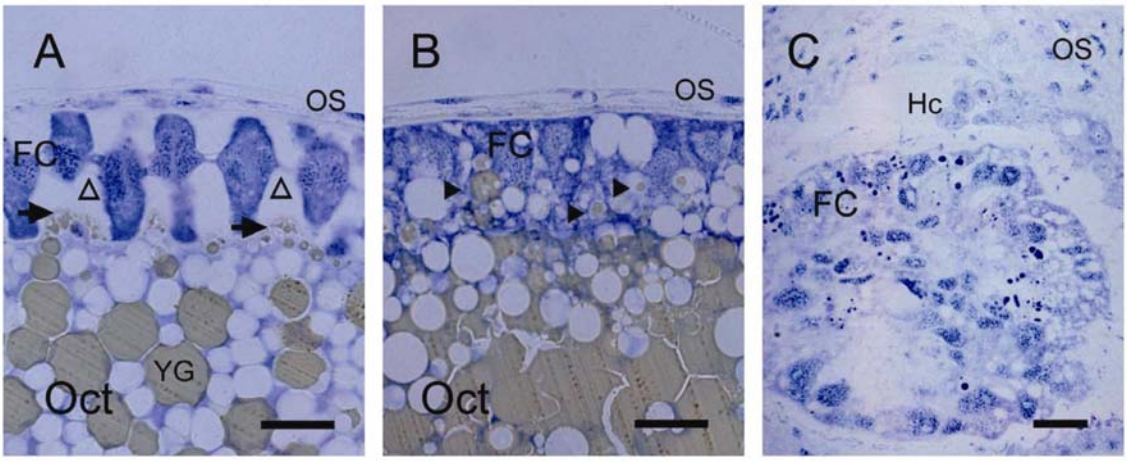

Fig. 3. Immunohistochemical detection of Vg in oocytes (Oct) and follicle cells (FC). A, Follicle undergoing normal vitellogenesis with yolk granules (YG) positive to anti-Vg serum. Brown areas represent the presence of the anti-Vg serum-positive materials. Arrows indicate small vesicles containing Vg formed as a result of endocytosis. Junctions connecting adjacent follicle cells (open triangles) were observed in intercellular spaces. B, Follicle during oosorption excised from a female starved for 4 days. Vg-positive materials phagocytosed in follicle cells (arrowheads) were observed. C, Follicle in the late stage of oosorption excise from a female starved for 6 days. Vg-positive materials were no longer detected in the follicle. Hemocytes (Hc) were observed in the space between the ovarian sheath (OS) and the degenerating follicle. The GMA resin section were 3-4 μm thick, and stained with DAB and toluidine blue. Scale bars: 20 μm. (Modified from Kotaki (2005) with permission of Balaban, Rehovot, Israel)

follicle cell layer was also digested (Data not shown). This indicated the presence of a low level of proteolytic activity in these sections.

Discussion

During oosorption, protein and Vg contents in the ovaries decrease. It is reported that Vg which has already accumulated in oocytes is released into the hemolymph during starvation-induced oosorption in the cockroach, *Periplaneta americana* (Bell, 1971). In general, Vg, which is synthesized by fat bodies in most insects, is transformed to Vn after uptake by oocytes (Raikhel and Dhadialla, 1992; Telfer, 2002). In *P. c. stali*, this transformation is accompanied by a change in subunit composition of Vg, as shown in Fig. 4, although Vg and Vn are immunologically identical (Kotaki, 2003). If oosorption-associated release of the yolk protein from oocytes takes place in this bug, the ovary-specific bands corresponding to Vn subunits that were recognized by the anti-Vg serum would had been found in the hemolymph of females during oosorption. However, Western blot analysis failed to detect such bands in this study. In *P. c. stali*, therefore, Vg is not released from the ovaries into the hemolymph. On the other hand, the immunohistochemical technique indicated the spreading of anti-Vg serum-positive materials throughout the cytoplasm of oocytes and their presence in the vacuoles in follicle cells in starved females. This suggests the breakdown of Vg-containing yolk granules and the uptake of Vg by follicle cells during oosorption. These results also imply that Vg and other proteins in the oocytes are taken up by the follicle cells,

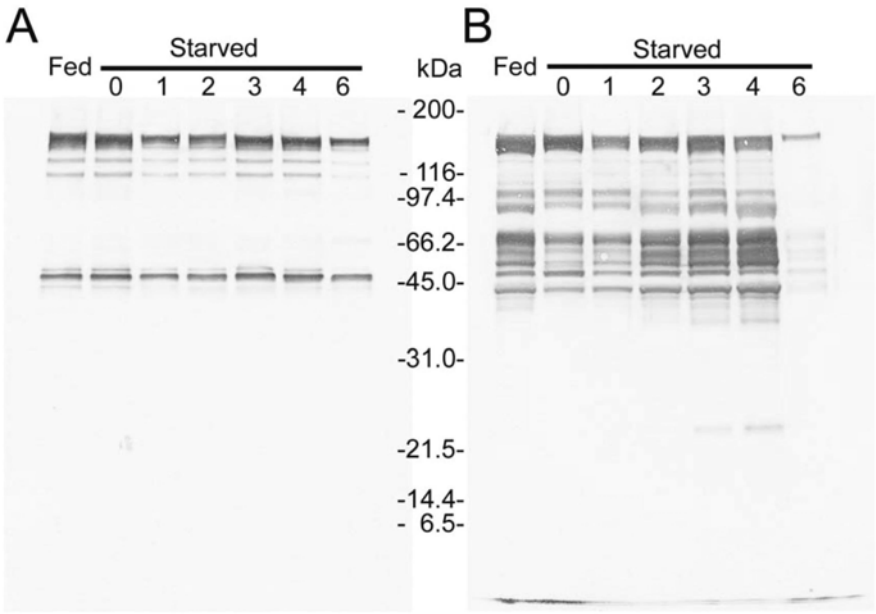

Fig. 4. Western blot analysis of hemolymph and ovary samples collected from females starved from day 4 onward. Samples resolved by SDS-PAGE were transferred to a PVDF membrane, and immunostained with anti-Vg serum. Numbers above the lanes indicate the length of the starvation period in days. Fed, samples collected from day 6-fed females. An aliquot of 0.02 μl of hemolymph or 0.02 ovary equivalents was applied to each lane, except for ovary extracts from fed females (to which 0.003 ovary equivalents was applied). (Modified from Kotaki (2003) with permission of Elsevier Science, Amsterdam, Netherlands)

degraded within these cells, and released into the hemolymph in the form of amino acids or small peptides. This possibility is, indeed, strongly supported by the result of FIZ demonstrating a high level of protease activity in the follicle cell layer in the ovaries during oosorption, in contrast to a very low activity in oocytes. Two processes have been suggested to be implicated in the mechanisms of oocyte degradation during oosorption (Bell and Bohm, 1975): phagocytosis by follicle cells, as observed in the locust, *Schistocerca gregaria* (Lusis, 1963), and lysis by lysosome-like bodies in the oocytes, as in the beetle, *Leptinotarsa decemlineata* (De Loof and Lagasse, 1970) and the bug, *Dysdercus similis* (Bhide, 1986). Both processes are known for the beetle, *Epilachna* (*Henosepilachna*) *vigintioctomaculata* (Kurihara, 1981; Kurihara, 1985). Uchida *et al*. (2001) have indicated that proteases in oocytes, which degrade yolk proteins during embryogenesis, are prematurely activated and play a role in proteolysis when the ovarian follicles are destined to atresia (oosorption) in the mosquito, *Culex pipiens pallens*. In *P. c. stali*, the follicle cells seem to play a more-important role in protein degradation than the oocyte cytoplasm does.

Follicle cells are multifunctional cells serving different tasks in the process of egg production; e.g., these cells regulate Vg uptake by providing a pathway for oocytes to access Vg in a juvenile hormone-dependent fashion during vitellogenesis, and they secrete the chorion in the final step of egg production (Raikhel and Dhadialla, 1992; Raikhel and Snigirevskaya, 1998). In *P. c. stali*, follicle cells appear to provide an additional function; i.e., a site

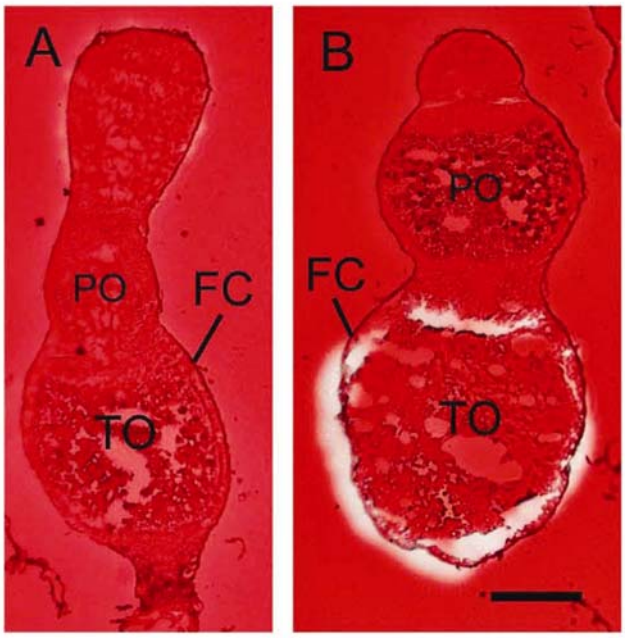

Fig. 5. Detection of protease activity in ovarioles excised from fed (A) and starved females for 4 days (B) by FIZ. White areas on a red background indicate sites where proteases were active. TO, terminal oocyte; PO, penultimate oocyte; FC, follicle cell. The frozen sections were 4 μm thick. Scale bar: 0.2 mm. (Modified from Kotaki (2005) with permission of Balaban, Rehovot, Israel)

of proteolysis during oosorption. Interestingly, these cells can change their function not only with the progress of oocyte development but also in a dichotomous way depending on the nutritional status or on certain internal factors.

The protease(s) responsible for protein degradation is (are) suggested to be of the cysteine protease type according to the results of experiments in which the effects of several protease inhibitors on proteolytic activity in the follicle cell layer were examined (Kotaki, 2005). However, purification and characterization of the protease(s) have not yet been carried out. We know almost nothing about how the protease is activated when oosorption is induced. In a very late stage of oosorption, follicle cells undergo apoptosis (Kotaki, 2005). It is important to determine the internal factors causing oosorption and the associated events such as apoptosis of follicle cells in this bug.

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褐翅綠椿象卵再吸收作用中卵黃原蛋白的命運及蛋白質崩解地點

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摘　要

當剝奪食物的供給，造成褐翅綠椿象卵細胞再吸收作用時，卵巢內的蛋白質及卵黃原蛋白會減少。在這個研究中，觀察卵黃蛋白是否會被完整地從卵巢釋放至血淋巴中，而不會在卵再吸收中被分解。西方墨點法利用抗卵黃原蛋白血清從卵巢正在發育雌蟲的血淋巴標本中顯現二條主要和許多不重要條斑，而卵巢萃取物在同樣的分析中反而呈現一套更複雜的條斑。因此卵黃蛋白很可能在卵細胞進行卵黃原蛋白累積之後才形成，西方墨點法無法從飢餓引起卵再吸收作用的雌蟲血淋巴樣本中偵測到卵再吸收或卵巢專屬胜肽片斷。另外，免疫組織染色技術發現，在此類雌蟲中，卵黃蛋白物質會被濾泡細胞吸收。利用原位酵素圖譜證明，在飢餓雌蟲的濾泡細胞內有高程度的蛋白酶活性，而在卵巢正在發育的雌蟲中，只偵測到低程度的活性。這些結果建議一旦卵再吸收作用被誘導出來，在卵細胞的卵黃原蛋白／卵黃蛋白會被濾泡細胞吸收及快速分解，結果造成胺基酸或小的胜肽可能被釋放於血淋巴中，由於太小以致於無法被抗卵黃原蛋白血清辨認。

關鍵詞：卵巢、卵黃蛋白、飢餓作用、蛋白質分解、椿象科。