Fluorescent Antibody Study of Chitobiase in Uninfected and Nuclear-Polyhedrosis-Virus Infected Larvae of the Silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

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(Received September 12, 1984)

Fluorescent antibody studies were conducted on the distribution of chitobiase in silkworm larvae, *Bombyx mori*, non-infected or infected with a nuclear polyhedrosis virus (NPV). Chitobiase, in non-infected control larvae, appeared first in the small tracheae and then in the large tracheae, anterior silk gland, foregut, hindgut, and integument in the process of larval-larval transformation. The enzyme rapidly disappeared when the larvae underwent ecdysis. A specific fluorescence of chitobiase was observed only on the chitinolytic tissues but not within their cells. In NPV-infected larvae, the distribution of chitobiase was similar to that in the control larvae, but the specific fluorescence was continuously observed when the larval ecdysis was blocked by the multiplication of NPV.

INTRODUCTION

When insects are invaded by pathogens, the larval ecdysis, pupation and eclosion are sometimes disturbed. The larvae of the silkworm, *Bombyx mori*, infected with the fungus, *Nomuraea prasina*, could not grow from the instar, at which the mark of fungus invasion appeared, to the next instar (KAWAKAMI, 1972); this apparently was because of the product of the fungus (MIKUNI and KAWAKAMI, 1975). Larvae of the rice stem borer, *Chilo suppressalis*, and the greater wax moth, *Galleria mellonella*, infected with *Chilo* iridescent virus, had prolonged larval stages without any signs of larval ecdysis. Moreover, these virus-infected larvae or pupae showed prothetely stages or supernumerary molts at the molting periods (ISHIKAWA and MUROGA, 1976). A similar observation was reported in larvae of the spruce budworm, *Choristoneura fumiferana*, infected with an insect poxvirus (BIRD, 1976). The infected larvae remained in the final instar for more than two weeks. In the silkworm larvae, the lethal time by a nuclear polyhedrosis virus (NPV) usually occurred prior to larval molt (KOBAYASHI et al., 1967).

Chitobiase, a chitinolytic enzyme, works only at larval molt, and its activity is especially high during the period from cuticle apolysis to larval ecdysis (KIMURA, 1977).

In the present study, we examined the presence of chitobiase in silkworm larvae infected with NPV, focusing our attention especially on its distribution in (1) larvae which showed no signs of larval molt and (2) chitinolytic tissues.

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MATERIALS AND METHODS

The F₁ silkworm hybrid of the cross, J.124 and C.124, was used. Fluorescent antibodies raised against the NPV and chitobiase were prepared as follows: The NPV was purified from hemolymph polyhedra of NPV-infected larvae by the method of KAWARABATA and MATSUMOTO (1973). An antiserum against the NPV was obtained from an immunized rabbit by the method of INOUE (1972). The rabbit was injected intravenously five times at 7 day-intervals with a concentration of purified NPV, corresponding to 7×10^9 polyhedra/ml. Chitobiase was purified from larval integuments at apolysis as previously described by KIMURA (1982) and the antiserum against the enzyme was prepared in the same manner as that for *Bombyx* α -N-acetylglucosaminidase (KIMURA, 1977). Injections in a rabbit were performed intravenously three times at 7 day-intervals, at levels of 1 mg of the purified enzyme protein. The method of preparing γ -globulin from each serum and conjugating the protein with fluorescent isothiocyanate was the same as reported by INOUE and AYUZAWA (1973).

Tissues were stained by direct immunofluorescence for the presence of NPV and chitobiase as follows: Second instar larvae shortly after ecdysis were fed NPV-treated leaves (polyhedron concentration 7.5×10^7 /ml) for 6 hr, and thereafter were fed fresh mulberry leaves. At 24 hr-intervals and up to 72 hr after ecdysis, samples of larvae were frozen as described previously (INOUE, 1972). Five larvae per test were examined. The frozen larvae were sectioned longitudinally at 10 μ m thickness using a DAMON/IEC cryostat microtome maintained at about -17°C . Specimens on a glass slide were fixed in acetone at 25°C for 10 min, and stained in an 80-fold diluted solution of the fluorescent anti-NPV γ -globulin or a 4-fold diluted solution of the fluorescent anti-enzyme γ -globulin for 60 min at 37°C . In order to avoid non-specific reaction, the specimens for enzyme detection were pretreated in 1% albumin at room temperature for 25 min. The specificity of fluorescence was checked by a blocking test. Observations were carried out with a fluorescent microscope, Tiyoda FM 200A.

RESULTS

Reaction of anti-chitobiase fluorescent antibody

The antiserum of chitobiase showed a precipitin band up to an 8-fold dilution against a homologous antigen from the larval integument in an agar immunodiffusion test. The titer was comparatively low, but the fluorescence of chitobiase was observed only in chitinogenous tissues and not in other tissues, e.g., the midgut in which the peritrophic membrane is composed of mucopolysaccharide and hyaluronic acid (NISHIZAWA et al., 1963).

Distribution of chitobiase at larval molting time in non-NPV-infected control larvae

In non-NPV-infected control larvae, the second instar larvae underwent apolysis at about 36 hr and exhibited the beginning of new cuticle formation at about 48 hr after the first ecdysis. Ecdysis occurred at about 60 hr after the first ecdysis. At 24 hr after the first ecdysis, only faint fluorescence of the enzyme appeared in the small tracheae, while it did not occur in other tissues. This indicated that the tracheal tube underwent apolysis before any other chitinogenous tissues. At 48 hr after the first ecdysis, the intensity of fluorescence became stronger and the distribution in tissues, except for the midgut,

Table 1. The appearance and distribution of a chitobiase and a nuclear polyhedrosis virus in silkworm larval tissues

Tissue	24-hr-old larva		48-hr-old larva		72-hr-old larva ^b	
	Enzyme	NPV	Enzyme	NPV	Enzyme	NPV
Non-infected larva						
Integument ^a	—	—	‡‡	—	—	—
Trachea	+	—	‡‡	—	—	—
Anterior silk gland	—	—	‡‡	—	—	—
Foregut	—	—	‡‡	—	—	—
Midgut	—	—	—	—	—	—
Hindgut	—	—	‡‡	—	—	—
NPV-infected larva						
Integument	—	—	‡‡	‡‡	‡‡	‡‡
Trachea	+	+	‡‡	‡‡	‡‡	‡‡
Anterior silk gland	—	—	‡‡	—	‡‡	—
Foregut	—	—	‡‡	—	‡‡	—
Midgut	—	—	—	+	—	+
Hindgut	—	—	‡‡	—	‡‡	—

NPV: nuclear polyhedrosis virus.

^a Level of fluorescence: ‡‡ > ‡ > + > — negative.^b Non-infected larvae ecdysed to third instar, but NPV-infected larvae remained in the second instar.

See the text for more details.

was wider than that observed at 24 hr (Table 1). The size patterns of fluorescence varied in the different tissues. The anterior silk gland exhibited homologous and lamellar fluorescence in the cuticular intima (Fig. 1A). The same pattern was seen in the inner layer of the endocuticle in the integument. In the trachea, different globular forms (0.7–2.9 μ m) of fluorescence were scattered (Fig. 1B). This difference in patterns might be due to the structure of the tissues since the tracheal tube has cuticular intima and taenidia with a helical pattern. At molt the digestive products including the chitinolytic enzyme that still remained at the top of the taenidia exhibited several fluorescent spheres. At 72 hr after the first ecdysis, the newly ecdysed larvae (third instar larvae) did not show any specific fluorescence in the tissues, suggesting that the chitobiase was no longer present. The fluorescence was not observed in any cell of the tissue examined at any specified time.

Distribution of chitobiase at larval molting time in NPV-infected larvae

In NPV-infected larvae, the first visible fluorescence specific for NPV appeared only in tracheal cells at 24 hr after the first ecdysis (see Table 1). At 48 hr after the first ecdysis, intense fluorescence was observed in the integument and trachea (Fig. 1C) and faintly in the midgut, but not in the anterior silk gland, foregut or hindgut. At this stage, no polyhedra were seen in any tissues. At 72 hr after the first ecdysis when the infected larvae did not show morphological signs of larval molting but had a bright skin, the distribution and intensity of the fluorescence was the same as that observed in 48-hr-old larvae. At this stage, polyhedra were found in tracheal cells (Fig. 1D).

On the other hand, the appearance and distribution of chitobiase in NPV-infected

Fig. 1. Micrographs showing immunofluorescent staining in tissues of second instar larvae of the silkworm, *Bombyx mori*. A: cross section of anterior silk gland showing the specific fluorescence due to chitobiase in the cuticular intima, but not in glandular cells. B: cross section showing globular fluorescences of different sizes due to chitobiase in the taenidia. C: longitudinal section of trachea showing specific fluorescence of the NPV in tracheal cells at 48 hr post virus inoculation. The fluorescence is greenish yellow and no polyhedra are detectable. The tracheal tubes show blue-colored autofluorescence. Arrow indicates the fluorescence specific for the NPV. D: longitudinal section of trachea showing fluorescence of a greenish-yellow color specific for chitobiase in the cuticular intima at 72 hr post virus inoculation. Polyhedra (blue) are scattered in the cells as spherical forms. Arrow indicates the fluorescence specific for chitobiase. Magnitude: $\times 980$.

larvae showed the same trend as that of the controls except in 72-hr-old larvae. Figure 1D shows the coexistence of chitobiase and NPV (polyhedra) in a trachea of a 72-hr-old larva. The NPV had developed in the tracheal cell, and the chitobiase was evident in the cuticular intima. The 72-hr-old infected larva exhibited intense fluorescence of the enzyme throughout most of its tissues. In such a larva, however, there was no new cuticular formation.

DISCUSSION

Specific fluorescence of chitobiase in non-NPV-infected control larvae was clear at the molt stage but was not distinctly observed during the intermolt stage. This observation confirmed those of a previous study regarding changes in the activities of chitinase and chitobiase in several tissues during metamorphosis (KIMURA, 1977). NOBEL-NESBITT (1963) reported, from electron micrographic observations of *Podura aquatica* at the larval molt, that large and granular secretions containing inactive enzymes appeared between the epidermal cell surfaces and the old cuticle. The enzymes became active at the time when these granules were divided into smaller spherical bodies after the comple-

tion of a new cuticle layer, and then parts of the cuticle were digested. Such granular secretions in the cuticle during the molting period were also found in *Calpodes ethlius* (LOCKE and KRISHNAN, 1973) and *Bombyx mori* (KIMURA et al., 1974). Thus, the existence of specific fluorescence suggests the possibility of active enzyme molecules of chitobiase.

According to KANEKATSU (1972, 1980), immunohistological observations and enzyme assay showed that *Bombyx* α -amylase was present as zymogen granules in midgut epithelial cells and was secreted into the lumen as active enzyme molecules. In our study, however, chitobiase was not detected at any time in the cells.

In NPV-infected larvae, the distribution of chitobiase in the NPV-infected trachea and in the non-infected anterior silk gland was the same as that in control larvae. However, the NPV-infected larvae, unlike the control larvae in which chitobiase disappeared rapidly after larval ecdysis, possessed high amounts of chitobiase (strong fluorescence) since the larvae did not undergo ecdysis. WATANABE and ARUGA (1971), in an experiment on the induction of artificial larval molt by Ponasteron A injection in NPV-infected larvae, found that when the larvae were inoculated with the NPV, the hypodermal cells prior to the formation of polyhedra were able to form a new cuticle during the molt, but the hypodermal cells which contained polyhedra were unable to form a new cuticle and the larvae could not undergo ecdysis.

The present experiment suggests that, in larvae which do not undergo ecdysis for whatever reason, the enzymes involved in larval molt, e.g., chitobiase, occur in large amounts suggesting high activity.

ACKNOWLEDGEMENTS

We sincerely thank Prof. Y. TANADA, Division of Entomology and Parasitology, University of California at Berkeley for his critical reading of the manuscript.

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