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SUPPRESSION OF NUCLEAR POLYHEDROSIS VIRUS INFECTION BY BETA-ECDYSONE IN THE SILKWORM, *BOMBYX MORI* L.

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Roger F. Hou and Jer-Chuan Yang (1989) Suppression of nuclear polyhedrosis virus infection by beta-ecdysone in the silkworm, *Bombyx mori* L. *Bull. Inst. Zool., Academia Sinica* **29**(1): 21-27. Suppression of *Bombyx mori* nuclear polyhedrosis virus (BmNPV) infection by β-ecdysone was studied using inoculation tests and enzyme-linked immunosorbent assay (ELISA). Mortalities caused by BmNPV were lowest by injecting the 5th instar larvae of the silkworm with β-ecdysone at 0.4 μg/larva before BmNPV inoculation. The larvae injected with various dosages of β-ecdysone had much lower ELISA values compared to those without hormonal injection, indicating that β-ecdysone is effective in suppressing BmNPV infection in *B. mori* L. Hormonal suppression of BmNPV infection is more predominant by inoculating with lower viral dosages.

Key words: *Bombyx mori*, BmNPV, β-ecdysone, ELISA.

Antiviral activity of the silkworm, *Bombyx mori*, to its nuclear polyhedrosis virus (BmNPV) has been suggested to be affected by production of antiviral proteins, insect endocrine secretions and ages as well as other environmental factors (Tanada, 1963; Watanabe, 1971; Aruga, 1973; Hayashiya 1978; Hou and Chiu, 1986; Liu and Hou, 1986; Hou *et al.*, 1988). Watanabe (1971) proposed that nutritional factors, *e.g.*, cholesterol, are rather important in susceptibility of silkworms to viruses because this steroil is a precursor for synthesizing ecdysone in insects (Downer, 1978). Aruga (1973) stated that insect hormones could alter cellular functions, division and differentiation, and therefore affected attachment, penetration and multiplication of insect viruses. Chen (1979) also indicated that cholesterol could reduce BmNPV multiplication and prolonged its incubation period when added to artificial diets. Keeley and Vinson (1975) obtained much lower larval mortalities by injecting β-ecdysone into *Heliothis virescens* following the inoculation with nuclear polyhedrosis virus (NPV) compared with injection with the Ringer's solution. Similar results were also obtained from injection with an ecdysteroid agent into *B. mori* (Liu and Hou, 1985). Antiviral activity of silkworm by administrations of ecdysteroids was reported to be due to reduction in antiviral red fluorescent protein (RFP) strength (Hou *et al.*, 1988). It seems that insect hormones play a role in regulation of viral infection in insect hosts.

The hypothesis as to whether ecdysteroids can inhibit BmNPV multiplication in silkworm larvae can be further confirmed by detecting the presence of virus using the enzyme-linked immunosorbent assay (ELISA) before or after administrations of pure ecdysteroids into the inoculated larvae because this technique proved to be useful for detecting several insect viruses in small quantities (Payment *et al.*, 1982; Shimizu, 1982). This study presents variations in ELISA reactions between ecdysteroid-treated and the Ringer's solution-injected silkworms after inoculating with BmNPV, and elucidates effect of ecdysteroids on reducing BmNPV infection in silkworms.

MATERIALS AND METHODS

Insects

The silkworm, *B. mori*, was a hybrid of (Kuo×Fu) × (Nung×Feng) raised by the Taiwan Sericultural Improvement Station. The larvae were fed on mulberry leaves at 25±1°C; 75±5% R. H. until the desired instar.

Virus source

BmNPV was originally isolated from diseased silkworms reared in sericultural farms of central Taiwan. Mass production of BmNPV was carried out by injecting the dissolved polyhedral solution into 5th instar larvae. The polyhedra were purified by centrifuging through a sucrose gradient, 30-80% (w/v) at 9,000 rpm (Beckman, L-40, using SW40Ti rotor) for 30 min, and washed several times with Tis-HCl (Tris-(hydroxymethyl)-methylamine) buffer (0.05 M, pH 7.4). The resultant pellet was frozen and dried.

Preparation of antiserum against BmNPV

Six mg of BmNPV powder was dissolved in 2 ml of 0.05 M NaCl+0.1 M Na_2CO_3 (pH 11.0) for 30 min and neutralized for 5 min with phosphate buffer to give pH 8.0-8.9. The virus solution was then centrifuged at 2,000 rpm for 20 min, its supernatant being filtrated through a 0.2 μ filter membrane. The filtrates were used as the antigen solution. The antiserum against BmNPV was obtained from intramuscular injections of the antigen solution into New Zealand white rabbits for 7 weeks. The booster injection was increased from 6 mg to 8 mg of antigen. The antibody titer was determined as the procedures described by Ball (1974) using a capillary (3×30 mm) as indicated by the opaque ring formation in the interphase of antigen and serum.

ELISA assay

The procedures were modified from Clark and Adams (1977), Lister (1978) and Crook and Payne (1980). The γ-globulin was separated from the antisera precipitated with saturated ammonium sulfate by using Sephadex G-25 - DE22 cellulose chromatography. The purified globulin was adjusted to OD 1.4 (=1 mg/ml). The enzyme-globulin conjugate was prepared with 5.4 mg alkaline phosphatase (Sigma type VII-S) suspended in 2 ml γ-globulin solution at 1 mg/ml. After dialyzing in phosphate buffered saline (PBS) for 18 hr, 1% glutaraldehyde was added to the enzyme-globulin solution to make up the final concentration of glutaraldehyde at 0.06% which was then further dialyzed overnight in PBS to eliminate glutaraldehyde. A bovine serum albumin solution at 5 mg/ml was then added to the conjugate.

The ELISA assays were carried out using a sandwich method. The γ-globulin was diluted with a coating buffer (1.5 g Na_2CO_3 and 2.93 g $NaHCO_3$ in 1 liter distilled water, pH 9.8) and coated at 200 μl in each well of a Nunc-Immuno Plate I. The

plate was washed with PBS-T (PBS+ 0.5% Tween-20) for 4 times. After drying, each well was added with 200 μl antigen samples (purified BmNPV or diseased silkworm tissue homogenates) and washed again with PBS-T for 4 times. Tissue homogenates of healthy silkworms or PBS-T were added as the controls. The enzyme conjugate was diluted with a conjugate buffer consisting of PBS-T plus 2% polyvinylpyrolidone and 0.2% ovalbumin. After washing, the substrate, *i.e.*, 0.8 mg/ml *p*-nitrophenyl phosphate, was added at 200 μl per well. The coloration was observed for 10-30 min and stopped with 50 μl of 3N NaOH. The plates were read at 405 nm using a micro-elisa reader (Dynatech MR 580) every 12 hr.

Ecdysteroid treatments

Solutions of β-ecdysone (Sigma Chemical Co.) at 0.2, 0.4 or 0.8 μg/μl were injected into newly molted 5th instar larvae. The controls were injected with the Ringer's solution. The β-ecdysone injected larvae were inoculated with BmNPV virions at 0.1, 1.0, 10 or 100 μg/larva by 24 hr post-injection. Larvae of each group of treatment were sampled once each 12 hr and their fat body tissues were dissected out for homogenizing as antigen samples for sandwich ELISA assays. In another series of tests, the 5th instar larvae were first inoculated with BmNPV and then injected with β-ecdysone. A total of 120 larvae in triplicate were tested in each treatment. Larval mortalities caused by NPV in both treatments were recorded and analyzed statistically.

RESULTS

Table 1 shows that mortalities caused by BmNPV were lowest by injecting the 5th instar larvae with β-ecdysone at 0.4 μg/larva either before or after viral inoculation. The incubation period of β-ecdysone injected larvae with BmNPV inoculation was usually 1-2 days longer than that of the uninjected larvae. Further evidence was obtained by detecting viral infection with ELISA assays. Figure 1 shows negative reaction of

Table 1
Mortality of 5th instar silkworms injected with different dosages of β-ecdysone and inoculated with 10 μg/larva BmNPV

β-ecdysone (μg/larva)	Mortality (%)	
	A	B
0	73.3 a	70.2 a
0.2	55.7 b	54.4 b
0.4	17.9 c	42.1 c
0.8	53.3 b	56.1 b

A: Injection with β-ecdysone first followed by BmNPV inoculation.
B: Inoculation with BmNPV first followed by β-ecdysone injection.
Means followed by the same letter are not significantly different at 5% level by the Duncan's multiple range test.

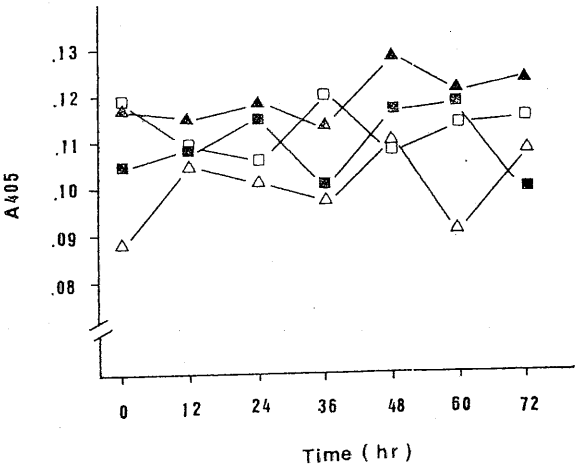

Fig. 1. The ELISA values of 5th-instar silkworms treated with different dosages of β-ecdysone at 12 hr interval. △—△:0; □—□: 0.2 μg/larva; ■—■: 0.4 μg/larva; ▲—▲: 0.8 μg/larva of β-ecdysone

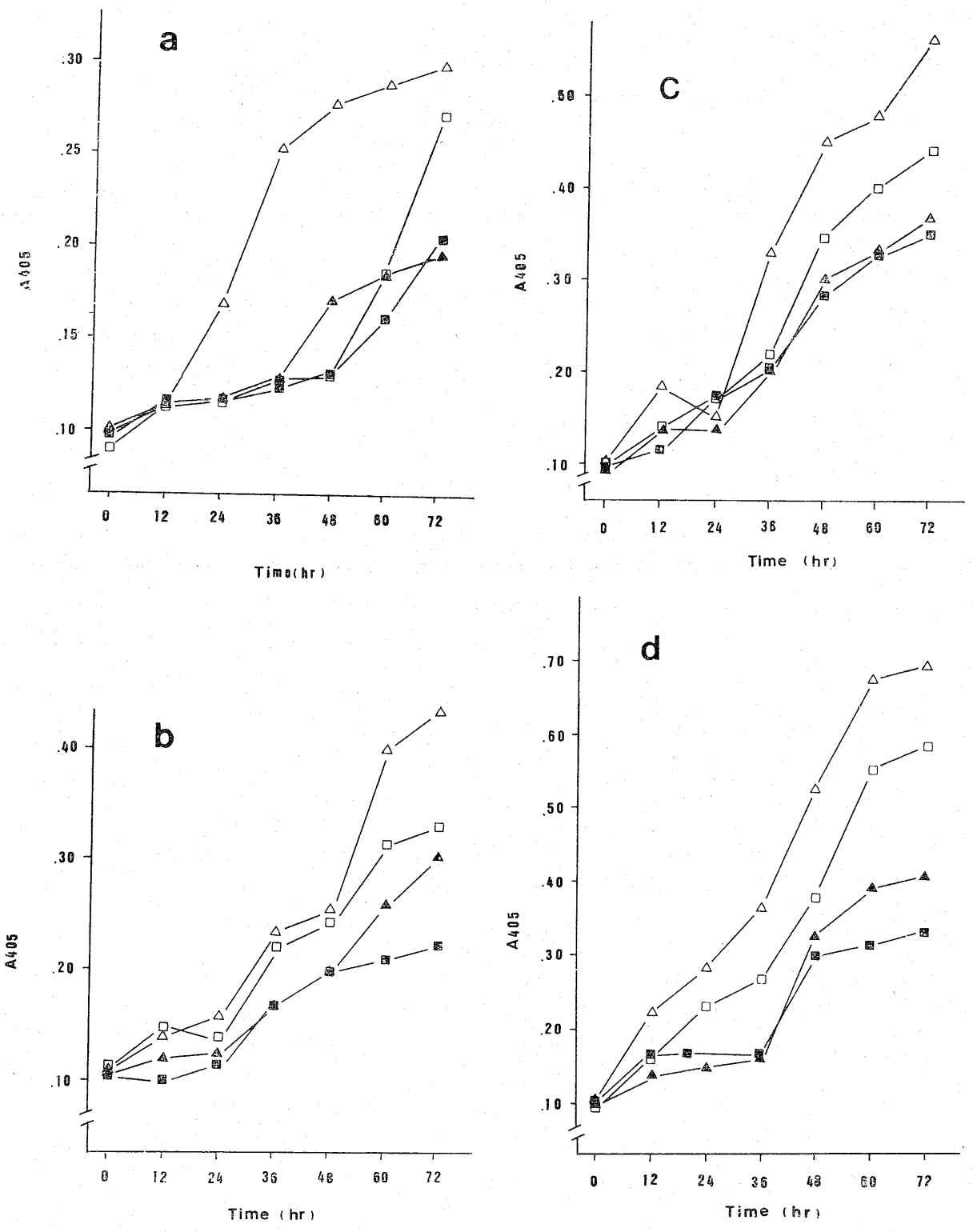

Fig. 2. The ELISA values of 5th instar silkworms injected with different dosages of β-ecdysone followed by inoculating with various dosages of BmNPV, a. 0.1; b. 1.0; c. 10; d. 100 μg/larva △—△: 0; □—□: 0.2 μg/larva; ▣—▣: 0.4 μg/larva; ▲—▲: 0.8 μg/larva of β-ecdysone

ELISA assays when injected the 5th intar larvae with β-ecdysone only without BmNPV inoculation as well as those of sham injection. In this case all larvae were as healthy as the normal insects without any injection. However, positive reaction could be obtained from the larvae inoculation with 0.1 μg/larva of BmNPV suspension, if detected 12 hr post-inoculation, whereas the larvae injected with various dosages of β-ecdysone 24 hr before inoculation with BmNPV did not show ELISA positive reaction until 48 hr post-inoculation (Fig. 2a). Inoculation of the larvae with BmNPV followed by β-ecdysone injection showed similar time course of ELISA reaction (Fig. 3). In addition, both Figures 2a-d and 3 showed that the β-ecdyson treated groups had lower ELISA values than those of BmNPV inoculation only.

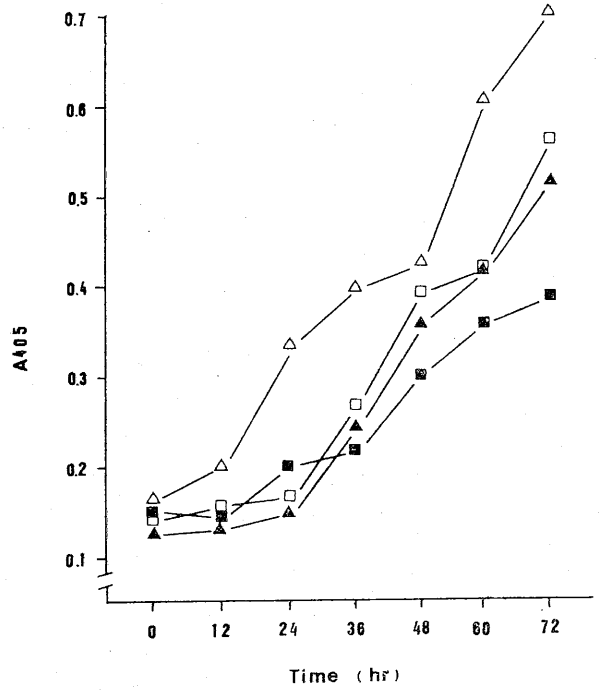

Fig. 3. The ELISA values of 5th instar silkworms inoculated with 10 μg/larva BmNPV followed by different dosages of β-ecdysone injections.
△—△: 0; □—□: 0.2 μg/larva; ■—■: 0.4 μg/larva; ▲—▲: 0.8 μg/larva of β-ecdysone

Beta-ecdysone was most effective in suppressing BmNPV at 0.4 μg/larva irrespective of the viral dosages inoculated; either lower or higher hormonal dosages than 0.4 μg/larva were poor for viral suppression (Figs. 2a-2d). The results showed that difference in ELISA values between β-ecdysone injected and uninjected larvae can be detected more predominantly using the sandwich ELISA assay when inoculated with a lower viral dosage rather than higher dosages (Figs. 2a, 2d). All treatments also revealed that the β-ecdysone treated groups delayed BmNPV infection.

DISCUSSION

Resistance of mammals to microbial infections can be accomplished by immune responses and hormonal regulation of physiological processes (Kass, 1960). In general there are similarities in hormonal actions between vertebrates and invertebrates. It is thus believed that the hormonal regulation of microbial infections could occur in insects as well. Inhibition of NPV infections by ecdysteroid agents has recently been reported in *B. mori* based on mortalities by viral inoculations (Liu and Hou, 1986). The present study further confirmed that the pure β-ecdysone either treated before or after viral inoculations is effective in reducing the larval mortality due to BmNPV infections.

The ELISA techniques have been used to detect various insect viruses with high sensitivity (Kelly *et al.*, 1978; Payment *et al.*, 1982; Shimizu, 1982). The sandwich ELISA assay was found to be useful for detecting BmNPV as low as 1 ng/ml while adding the purified virions as antigens (Yang, 1984). Therefore this method is reliable for distinguishing antiviral activity of ecdysteroids at low viral concentrations in insect tissues.

The present results clearly showed suppression of BmNPV formation in ecdysteroid-injected silkworm larvae compared with those insects without β-ecdysone injection. This further confirmed effectiveness of ecdysteroids on suppression of baculovirus infections in insects. Our findings were further supported by *in vitro* studies that treatment of *Estigmene acrea* cells with β-ecdysone after inoculating with a *Tipula* iridescent virus may inhibit capsid formation, resulting in the absence of normal virion assembly as seen in electron micrographs (Kloc *et al.*, 1984). Keeley and Vinson (1975) proposed that enhancement of protein and RNA syntheses in host cells by ecdysteroids could elicit competition for nucleotides and amino acids with NPV and thus suppresses viral multiplication in *Heliothis virescens*. It is not known whether β-ecdysone inhibits BmNPV infection in the ways as mentioned above. However, the fact that much lower ELISA reactions were detected after injecting *B. mori* larvae with β-ecdysone is suggestive of suppression of BmNPV multiplication in the silkworm by exogenous administration of ecdysteroids *in vivo*.

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REFERENCES

Aruga, H. (1973) *Introduction to Insect Pathology.* Yokendo Ltd., Tokyo, Japan. 614pp. (In Japanese)

Ball, E. M. (1974) *Serological tests for the identification of plant viruses.* The American Phytopathological Society, Plant Virology Committee. 31 pp.

Chen, R. S. (1979) Studies on nucleopolyhedrosis of the silkworm, *Bombyx mori* L. and factors affecting its pathogenicity. M. S. Thesis, National Chung Hsing University, 74pp. (In Chinese)

Clark, M. T. and A. N. Adams (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant virus. *J. Gen. Virol.* **34**: 475-483.

Crook, N. E. and C. C. Payne (1980) Comparison of three methods of ELISA for baculoviruses. *J. Gen. Virol.* **46**: 29-37.

Downer, R. H. (1978) *Functional role of lipids in insects.* In "Biochemistry of Insects" (M. Rockstein, ed.), Academic Press, N. Y. pp. 58-92.

Hayashiya, K. (1978) Red fluorescent protein in the digestive juice of the silkworm larvae fed on host-plant mulberry leaves. *Entomologia exp. appl.* **24**: 228-236.

Hou, R. F. and C. S. Chiu (1986) Antiviral proteins in midgut of the silkworm, *Bombyx mori*, fed on different food sources. *Entomologia exp. appl.* **42**: 3-8.

Hou, R. F., C. S. Chiu and M. L. Tsai (1988) Effect of ecdysterone on red fluorescent protein intensity in the silkworm, *Bombyx mori* L. *Bull. Inst. Zool., Academia Sinica* **27**: 1-6.

Kass, E. H. (1960) Hormones and host resistance to infection. *Bacteriol. Rev.* **24**: 177-185.

Keeley, L. L. and S. B. Vinson (1975) β-ecdysone effects on the development of nucleopolyhedrosis in *Heliothis* spp. *J. Invertebr. Pathol.* **20**: 121-123.

Kelly, D. C., M. L. Edwards, H. F. Evans and J. S. Robertson (1978) The use of enzyme-linked immunosorbent assay to detect a nuclear polyhedrosis virus in *Heliothis armigera* larvae. *J. Gen. Virol.* **40**: 465-469.

Kloc, M., P. E. Lee and S. Tajbakhsh (1984) The effect of two insect hormones on the replication of *Tipula* iridescent virus in *Estigmine acrea* cells in suspension culture. *J. Invertebr. Pathol.* **43**: 114-121.

Lister, R. M. (1978) Application of the enzyme-linked immunosorbent assay for detecting viruses in soybean and plants. *Phytopathology* **68**: 1393-1400.

Liu, H. S. and R. F. Hou (1985) Studies on inhibition of infection with nuclear polyhedrosis virus by an ecdysteroid-containing agent in the silkworm, *Bombyx mori* L. *Chinese J. Entomol.* **5**: 23-30.

Payment, D. J., S. Arora and S. Belloncik (1982) An enzyme-linked immunosorbent assay for the detection of cytoplasmic polyhedrosis virus. *J. Invertebr. Pathol* 40: 55-60.

Shimizu, S. (1982) Enzyme-linked immunosorbent assay for the detection of the flacherie virus of the silkworm, *Bombyx mori*. *J. Sericult. Sci. Japan*, 51: 370-373. (In Japanese)

Tanada, Y. (1963) *Epizootiology of infectious diseases.* In "Insect Pathology, An Advanced Treatise" (E. Steinhaus, ed.), Vol. 2, Academic Press, N. Y. pp. 423-475.

Watanabe, H. (1971) *Pathophysiology of cytoplasmic polyhedrosis in the silkworm.* In "The Cytoplasmic Polyhedrosis Virus of the Silkworm" (H. Aruga and Y. Tanada, eds.). University of Tokyo Press, Japan. pp. 151-184.

Yang, J. C. (1984) Studies on the enzyme-linked immunosorbent assay (ELISA) for detection of nuclear polyhedrosis virus in the silkworm, *Bombyx mori* L. M. S. Thesis, National Chung Hsing University, 63pp. (In Chinese)

昆蟲脫皮素抑制家蠶核多角體病毒之感染

侯豐男　楊哲權

昆蟲脫皮素 (β-ecdysone) 抑制家蠶核多角體病毒 (BmNPV) 之感染，經採用接種試驗及酵素結合免疫吸附檢定法 (ELISA) 加以研究。家蠶罹病死亡率以 0.4 μg/larva 之脫皮素在 BmNPV 接種前注入五齡幼蟲者最低。將不同劑量之脫皮素注入幼蟲均較未注入激素之蟲體，具更低之 ELISA 值，此結果顯示脫皮素有抑制 BmNPV 感染家蠶之效果。激素抑制病毒感染之效應，以接種低量病毒時較爲顯著。