Formosan Entomologist
Journal Homepage: entsocjournal.yabee.com.tw

【Research report】

脫皮素熟蠶劑抑制家蠶核多角體病毒感染之研究【研究報告】

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Received: Accepted: Available online: 1985/03/01

Abstract

摘要

Mayuran® 係一種含脫皮素之熟蠶劑商品，通常施用於第五齡家蠶 (Bombyx mori L.) ，促使其結繭時間一致。若將此劑以0.4mg/ml 之濃度施於四齡起蠶，經24小時後再接種核多角體病毒 (NPV) ，則可抑制 NPV 之感染。五齡起蠶經 Mayuran® 處理12小時候，再接種 NPV 即可抑制病毒感染。反之，若先接種 NPV，再處理熟蠶劑，便無法減少，因為NPV 會引起罹病死亡率。 分析血液中可溶性蛋白質之含量得知，經熟蠶劑處理後，再接種NPV之蠶體，其所含此種蛋白質之量與未經任何處理者相似。以SDS-polyacrylamide gel 電泳法分離家蠶血液蛋白的種類，至少可得17種不同分子量之蛋白質。分離的結果顯示用熟蠶劑處理，再接種 NPV，經126小時後，此家蠶的血液所含之病毒蛋白會被抑制。由本試驗結果，可以推測脫皮素在接種 NPV 之前處理，可抑制家蠶核多角體病之發生。

Key words:
關鍵詞:

Full Text: PDF(5.36 MB)

中華昆蟲 5 : 23– 30 (1985)
Chinese J. Entomol. 5 : 23– 30 (1985)

STUDIES ON INHIBITION OF INFECTION WITH NUCLEAR POLYHEDROSIS VIRUS BY AN ECDYSTEROID-CONTAINING AGENT IN THE SILKWORM *BOMBYX MORI* L.

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ABSTRACT

An ecdysteroid-containing agent, *viz.*, Mayuran® which is conventionally used for regulating cocooning of 5th-instar larvae of the silkworm, *Bombyx mori* L., was found to be effective in suppressing infection of nuclear polyhedrosis virus (NPV) when applied to the 4th-instar larvae at 0.4 mg/ml, 24 hr prior to the virus inoculation. Mayuran® might exert suppression of NPV incidence if applied to the 5th-instar larvae 12 hr before the virus inoculation. However, this agent did not reduce the mortality caused by NPV if treated after the virus inoculation. Analysis of the hemolymph soluble protein showed that the Mayuran® -treated silkworms, which were later inoculated with NPV, had the contents similar to those of normal ones without Mayuran® and NPV treatments. The electrophoretic separation using SDS-polyacrylamide gel from silkworm hemolymph showed at least 17 protein bands. Treatment of the 4th-instar larvae with Mayuran® prior to NPV inoculation resulted in inhibition of some viral proteins in hemolymph after 126 hr. This study proposed that ecdysteroids might be capable of suppressing the development of nucleopolyhedrosis in *B. mori* when treated before NPV inoculation.

INTRODUCTION

It is generally believed that insect hormones play an important role in regulation of various physiological functions. Subrahmanyam and Ramakrishnan (1980) reported that the activity of copora allata was induced in the larvae of *Spodoptera litura* infected with a baculovirus, and their juvenile hormone titer went down two days behind the healthy insects. Keeley and Vinson (1975) pointed out that replication of nuclear polyhedrosis virus (NPV) in *Heliothis virescens* was reduced after injecting with β-ecdysone. Riddiford (1975) also stated that hormones and their analogues could enhance an insect host to inhibit the growth of its pathogens. Therefore it is conceivable that there is direct or indirect relationship between pathogens and hormones in insects.

Infection of NPV in the silkworm, *Bombyx mori* L. is affected by some biotic and abiotic factors (Chen and Hou, 1980). In the present study, we investigated effect of a cocooning regulator, Mayuran® , which contains ecdysterone, on infectivity of NPV in the silkworm.

MATERIALS AND METHODS

Insects and virus inoculum

The silkworm *B. mori*, is a hybrid of (Kuo x Fu) x (Nung x Feng), provided by the Taiwan Sericultural improvement Station. All the larvae tested were fed on mulberry leaves. The NPV was purified according to the procedures of Hou and Chao (1980), and the virus inocula were prepared by suspending the purified virus powder in Tris buffer (pH 7.4, 0.05 M) to make up

two concentrations, i.e., 2.5 x 10^6 and 6.5 x 10^6 PIB/ml.

Treatment of Mayuran®

Mayuran® is a product of the Ikeda Chemical Co. Japan., and was given by the local agent. Its active ingredient is ecdysterone at a concentration of 1 mg/g.

Mayuran® was diluted with sterile distilled water to the following concentrations: 0.08, 0.10, 0.13, 0.20 and 0.40 mg/ml. Mulberry leaves were soaked into each dilution and dried in the air. Then three replications totalling sixty 4th-instar larvae were fed on these leaves and were inoculated with NPV at 2.5 x 10^6 PIB/ml by feeding on the virus-contaminated leaves after 24 hr. The controls were fed on the leaves smeared with sterile distilled water. In another experiment, the larvae were first inoculated with NPV followed by treatment with Mayuran® after 24 hr. For the 5th-instar larvae, the intervals of Mayuran® treatment and NPV inoculation or *vice versa* were 12, 36 and 60 hr. The Mayuran® concentration was 0.5 mg/ml, and the NPV suspension contained 6.5 x 10^6 PIB/ml.

Analysis of soluble protein in hemolymph

Lowry's method using the folin-phenol reagent was employed to measure the protein contents of hemolymph in the 4th-instar larvae (Lowry *et al.*, 1951). A sample of 0.02 ml hemolymph collected from five larvae was analyzed. Three replications were carried out in each treatment.

For determining the electrophoregram of the silkworm hemolymph, an aliquot of 0.15 ml blood sample was centrifuged at 4,000 rpm for 30 min, and separated by the slab acrylamide gel electrophoresis according to the Laemmli's method (Laemmli, 1970). Electrophoretic separation was conducted using a 12.5 x 16 x 0.2 cm slab gel containing 11% polyacrylamide and 1% sodium dodecyl sulfate (SDS), running at 150 V for 3 hr at 4°C. The gel was stained with coomassie brilliant blue R. The protein molecular weight was estimated by the method of Weber and Osborn (1969). The Sigma product "MW-SDS-70" which is composed of albumin, albumin egg, pepsin, trypsinogen, β-lactoglobin and lysozyme was used as the molecular weight marker.

RESULTS AND DISCUSSION

Table 1 shows that the silkworms did not succumb to NPV infection after feeding on the mulberry leaves treated with 0.4 mg/ml Mayuran® . In contrast, the larval mortality caused by NPV was ca. 40%, being higher than that of any other groups. From these results, we presume that ecdysterone in Mayuran® seems to be able to enhance the 4th-instar larvae of *B. mori* against NPV infection if treated before virus inoculation. Keeley and Vinson (1975) proposed that ecdysone may stimulate the metabolism of the host cells, increasing the cell's competitiveness for available nucleotides and amino acids, and can thus retard the development of the infective virus in *H. virescens.* Whether these physiological phenomena take place in *B mori* remain uncertain. Because it is difficult to measure the critical point of Mayuran® concentration, we can not find the linear correlation between dosage and mortality yet.

As can also be seen in Table 1, the larval mortality was ca. 19% or more, resulting from various concentrations of Mayuran® which were treated 24 hr after NPV inocluation. This result indicates that ecdysterone is not markedly effective in suppressing NPV infection when treated after virus inoculation, revealing that it cannot be used as a chemotherapeutic agent, although Chen (1979) indicated that cholesterol, a precursor of ecdysone, might prolong the NPV

incubation period, if added to an artifical diet at 0.5-1.0 g/100g.

Table 1. The larval mortality of 4th instar *Bombyx mori* treated with Mayuran® and NPV[1]

Conc. of Mayuran® (mg/ml)	Percent Mortality (Means ± SD)[3]	
	Treatment	
	Mayuran®, then NPV	NPV, then Mayuran®
0.00	40.00 ± 5.00 a	33.33 ± 5.77 a
0.08	6.67 ± 2.89 b	30.00 ± 0.00 a
0.10	8.33 ± 2.89 b	21.67 ± 2.89 bc
0.13	5.00 ± 0.00 b	19.33 ± 5.00 bc
0.20	6.67 ± 2.89 b	36.67 ± 7.64 a
0.40	0.00 c	35.00 ± 5.00 a
Check[2]	0.00 c	0.00 b

1. NPV (2.5 x 10^6 PIB/ml) inoculated 24hr after Mayuran® application, Mayuran® applied 24hr after NPV (2.5 x 10^6 PIB/ml) inoculation; 20 larvae/replicate, 3 replications
2. Without NPV and Mayuran® treatment
3. Means followed by the same letter are not significantly different at 5% level according to Duncan's multiple range test, SD: standard deviation.

Treatment of the 5th-instar larvae with Mayuran® at a higher concentration revealed that the mortality caused by NPV was not significantly different between uninoculated larvae and those inoculated 12 or 60 hr after Mayuran® treatment while the larvae inoculated with NPV after 36 hr had a mortality as high as that of inoculation with NPV only, and that the larvae received Mayuran® after NPV inoculation did not show reduction in mortality (Table 2). Obviously, activity of the ecdysterone in Mayuran® against NPV in vivo is diminished 36 hr after treatment. The low mortality occurred in the larvae which were inoculated with NPV after 60 hr could be ascribed to their approach to pupation, exhibiting the maturation immunity as proposed by Tanada (1963). Calculation of the percentage of cocoon layer from the survivors showed that Mayuran® can enhance the silk production to some extent after suppression of the NPV infection (Table 2). From these results, it is further believed that the ecdysterone-containing agent is applicable for prevention of the silkworm nucleopolyhedrosis in addition to its general usage as the cocooning regulator.

To study the possible mechanisms of Mayuran® treatment against NPV infection, we analyzed the soluble protein content and electrophoregrams in tested silkworms. Fourth-instar larvae of *B. mori* were treated with 0.4 mg/ml Mayuran®, followed by inoculating with NPV after 12 hr. Figure 1 shows that the healthy larvae have higher soluble protein content than the NPV-inoculated silkworms. Within the first 24 hr after Mayuran® treatment, the protein content was similar

Table 2. The larval mortality and cocoon layer of 5th instar *Bombyx mori* treated with Mayuran® and NPV[1]

Treatment	Mortality (%)	Cocoon layer (%)
	Means ± SD[3]	Means ± SD[3]
Mayuran® only	0.00 b	26.67 ± 1.21 a
Mayuran®, 12hr NPV	5.00 ± 0.00 b	26.17 ± 3.35 a
Mayuran®, 36hr NPV	48.33 ± 2.89 a	14.79 ± 0.28 b
Mayuran®, 60hr NPV	5.00 ± 0.00 b	26.49 ± 1.80 a
NPV, 12hr Mayuran®	36.67 ± 2.89 a	23.03 ± 6.62 a
NPV, 36hr Mayuran®	36.67 ± 5.76 a	24.19 ± 4.37 a
NPV only	38.33 ± 7.63 a	23.83 ± 2.97 a
Check[2]	0.00 b	24.49 ± 2.50 a

1. Mayuran® conc.: 5mg/ml; NPV conc. : 6.5 x 10^6 PIB/ml; 20 larvae/replicate, 3 replications
2. Without Mayuran® and NPV treatment
3. Means followed by the same letter are not significantly different at 5% level, SD: standard deviation

irrespective of different treatments. Later, the protein content of healthy and Mayuran® - NPV - treated larvae became higher than that of NPV-inoculation *B. mori*. The protein content in the blood collected from the Mayuran® - NPV - treated larvae was markedly higher than that of healthy larvae after 110 hr of treatment. Before ecdysis, the healthy and Mayuran® - NPV - treated larvae have a similar protein content, which is much higher than that of NPV-infected larvae. Martignoni and Milstead (1964) reported that fat bodies of noctuid larvae can be destroyed in the course of NPV infection, resulting in hypoproteinemia in hemolymph. Watanebe and Kobara (1971) also found this pathological change in *B. mori*. From our results, it was found that after Mayuran® treatment, the protein content in the silkworm hemolymph was elevated appreciably compared with the NPV-inoculated larvae. The increase in the protein content may possibly contribute to resistance of the silkworm to the NPV infection.

Using SDS-polyacrylamide gel electrophoresis, we separated the hemolymph proteins from the normal, NPV-inoculated, and Mayuran® NPV - treated silkworms. Figure 2 shows that at least seventeen hemolymph proteins were resolved with molecular weight from 2.5-8.1 x 10^4. Nakasone and Kobayashi (1965) reported that only six proteins were separated from the 4th-instar silkworms by disc acrylamide gel electrophoresis. It is obvious that we obtained more detailed and accurate protein bands from this separation.

The electrophoretic separations of the hemolymph proteins from the NPV-infected larvae were similar to those from the Mayuran® -NPV-treated larvae and the controls from 12 to 36 hr after Mayuran® treatment (Fig. 3). Watanabe (1967) also found the electrophoretic patterns to be similar when separated from healthy and NPV-infected larvae of the fall webworm, *Hyphantria cunea* in the early stage of infection. In Mayuran® and NPV treated larvae, the protein band 6.7

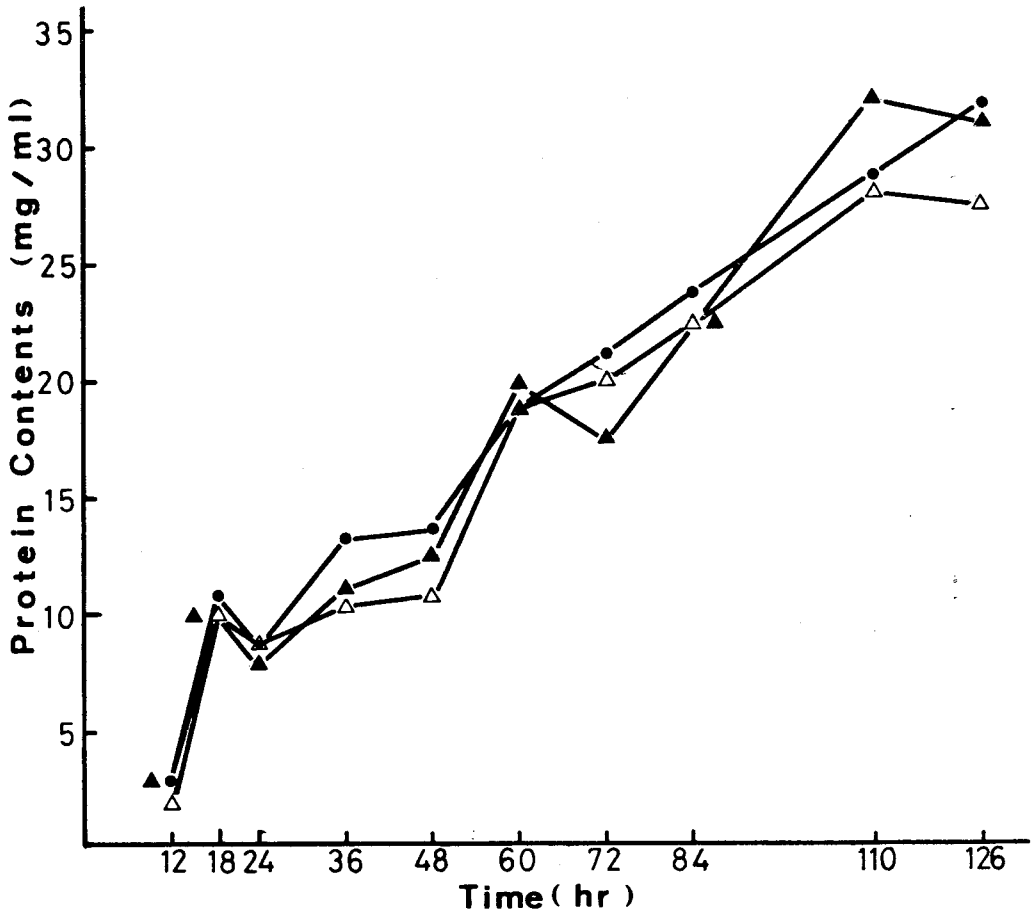

Fig. 1. Protein contents in hemolymph of 4th-instar larvae of *Bombyx mori* after various treatments. ● : Check; ▲: Mayuran® treatment and NPV inoculation; △: NPV inoculation only.

was scarcely visible at 48 hr (Fig. 3), and the protein bands 6.7, 6.8, and 7.1 disappeared completely by 60 hr after treatment, but several new bands below 6.2 were visible at the same time (Fig. 4). Kobayashi and Kawase (1980, 1981) reported that ecdysterone may activate host metabolism and play an active role in NPV development in isolated pupal abdomens. Kozlov and Sidorova (1975) calculated the molecular weight of matrix proteins of *B. mori* NPV to be 2.0-2.8 x 10^4. Therefore, it seems that those bands below 6.2 which are produced in the NPV-inoculated larvae could be viral proteins. These bands have become distinct in the NPV-inoculated larvae since 110 hr after inoculation, whereas they tend to diminish in the NPV-inoculated larvae treated with Mayuran® after 126 hr (Fig. 4). Meanwhile, the protein bands with molecular weight of 6.8-7.1 x 10^4 were destroyed in the NPV-inoculated larvae after 84 hr of treatment, but they remained intact in the uninoculated larvae (Fig. 4). Riddiford (1975) stated that hormones could enhance an insect host to inhibit the growth of its pathogens. Evidently, the ecdysteroids may inhibit the production of viral proteins in host tissues, and thus suppress the development of nucleopolyhedrosis in the silkworm when treated prior to inoculation. Effect of hormones on changes of electrophoretic protein patterns in other tissues is undertaking in this laboratory.

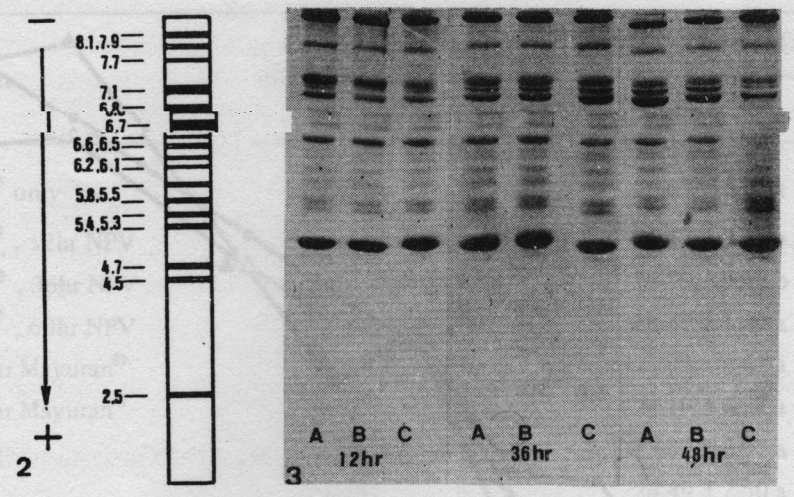

Fig. 2. The electrophoretic protein separation from hemolymph of 4th-instar *Bombyx mori*. Each band marked with the calculated molecular weight (X 10^4).

Fig. 3. The electrophoretic separation of hemolymph protein of 4th-instar *Bombyx mori* 12-48 hr after various treatments.
A: Check (without NPV and Mayuran® treatments). B: NPV inoculation only.
C: Mayuran® treatment and NPV inoculation.

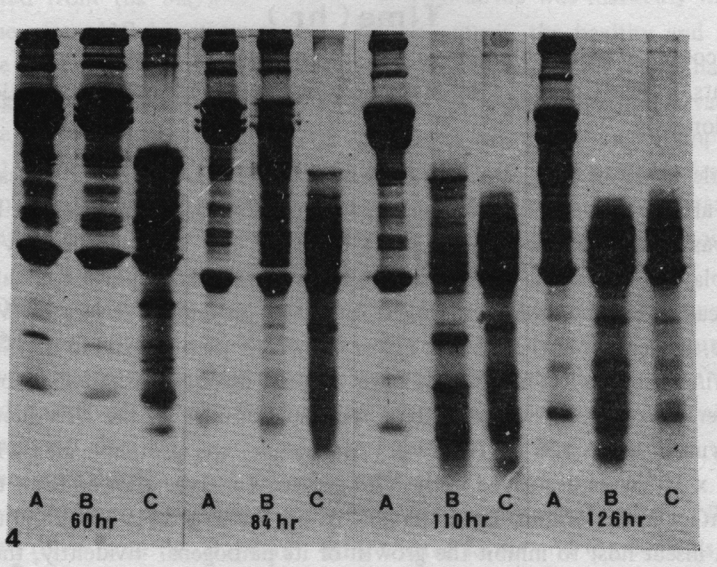

Fig. 4. The electrophoretic separation of hemolymph protein of 4th-instar *Bombyx mori* 60-126 hr after various treatments.
A: Check (without NPV and Mayuran® treatments). B: NPV inoculation only.
C: Mayuran® treatment and NPV inoculation.

ACKNOWLEDGEMENTS

This work was supported by research grant number NSC71-0409-B005-03 from the National Science Council, ROC. We are indebted to the Taiwan Sericultural Improvement Station for supplying the silkworm eggs, and to Dr. W. C. Chang and Mr. J. Tso, Institute of Biochemistry, Academia Sinica, Taipei, Taiwan, ROC, for their technical assistance.

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脫皮素熟蠶劑抑制家蠶核多角體病毒感染之研究

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Mayuran® 係一種含脫皮素之熟蠶劑商品，通常施用於第五齡家蠶（*Bombyx mori* L.），促使其結繭時間一致。若將此劑以 0.4 mg/ml 之濃度施於四齡起蠶，經 24 小時後再接種核多角體病毒（NPV），則可抑制 NPV 之感染。五齡起蠶經 Mayuran® 處理 12 小時候，再接種 NPV 即可抑制病毒感染。反之，若先接種 NPV，再處理熟蠶劑，便無法減少，因爲 NPV 會引起罹病死亡率。

分析血液中可溶性蛋白質之含量得知，經熟蠶劑處理後，再接種 NPV 之蠶體，其所含此種蛋白質之量與未經任何處理者相似。以 SDS-polyacrylamide gel 電泳法分離家蠶血液蛋白的種類，至少可得 17 種不同分子量之蛋白質。分離的結果顯示用熟蠶劑處理，再接種 NPV，經 126 小時後，此家蠶的血液所含之病毒蛋白會被抑制。由本試驗結果，可以推測脫皮素在接種 NPV 之前處理，可抑制家蠶核多角體病之發生。