Promotion of nucleopolyhedrovirus infection in larvae of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) by an antibiotic, nikkomycin Z

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Abstract

Nikkomycin Z, an antibiotic, promoted infection of *Bombyx mori* nucleopolyhedrovirus (BmNPV) in 4th-stadium *B. mori* larvae, when the chemical was incorporated into the larval diet. The median lethal dose (LD₅₀) of BmNPV was decreased as the concentration of nikkomycin Z was increased. LD₅₀ of BmNPV in larvae that received 10 ppm nikkomycin Z was 0.7–1.1 polyhedra/larva. The peritrophic membrane was not observed in the gut of larvae that had received 10 ppm nikkomycin Z. Although the growth of larvae that had received 10 ppm nikkomycin Z was delayed, they molted to the next stadium.

Key words: Nikkomycin Z, chitin synthesis inhibitor, nucleopolyhedrovirus, *Bombyx mori*, microbial pesticide

INTRODUCTION

Insect viruses such as nucleopolyhedroviruses (NPVs) are promising candidates for pest controlling agents (Shieh, 1989), and several NPVs have been registered as microbial pesticides (see Okada, 1994). For commercial production of the virus, cost of the viral insect control must be competitive with that of existing chemical insect controls. The cost of producing a viral product using a large amount of hosts is high, hampering industrial production of viral pesticides. As a result, Sandoz discontinued virus production in 1983 (see Sato, 1985; see Shapiro and Vaughn, 1995).

There are many reports of chemicals that enhance viral infection (see Goto, 1999), which aim to reduce the viral dose that kills target insects; the goal is to require viral pesticide production on a smaller scale. Arakawa (2002) showed that the silkworm, *Bombyx mori*, ingesting acetone-dissolved flufenoxuron became sensitive to *B. mori* NPV (BmNPV). Flufenoxuron is an insect growth regulator (IGR) that inhibits epidermal chitin synthase, resulting in an inability to molt (Lee et al., 1990). Effectiveness of flufenoxuron suggests that chitin-related chemicals are candidates for an enhancer of viral infection. In the present paper, we report that a chitin synthesis inhibitor, nikkomycin

Z, promotes NPV infection in the silkworm, *B. mori*.

MATERIALS AND METHODS

Insects and virus. Silkworm larvae (Fuyoh×Tohkai) were reared similarly to the method described in Arakawa (2002). Aliquots from the same polyhedral suspension of BmNPV strain A 505 as had been used in Arakawa (2002) were used in this study, and contained 7.4×10^8 polyhedra/ml.

Chemicals. Nikkomycin Z, an antibiotic, purchased from Sigma Chemical Co., Ltd. was tested for the promotion of viral infection and was compared with the effect of flufenoxuron purchased from Wako Pure Chemical Industries Co., Ltd. These chemicals were administered perorally by incorporating them into the diet as follows. Nikkomycin Z was dissolved in 1 ml distilled water and mixed with 19 g crushed Silkmate 2S (Nihon Nohsan-Kogyo Co., Ltd.), an artificial diet. The mixture was kneaded well with a medical spurtle. Flufenoxuron was dissolved in 400 μ l acetone and mixed with 19 g crushed Silkmate 2S and 600 μ l distilled water, and the mixture was kneaded well. This diet contained 2% acetone immediately after preparation. The final concentrations of nikkomycin Z and flufenoxuron were 1–100 ppm and 10–100

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ppm, respectively. Control larvae received a similarly prepared diet without nikkomycin Z and flufenoxuron.

Bioassay for NPV susceptibility. The polyhedral suspension was serially diluted tenfold with distilled water and used for preparation of viral paste, which consisted of dried mulberry leaf powder (0.1 g), 10% (w/w) sucrose solution (100 μ l) and BmNPV polyhedral suspension (150 μ l). This paste was kneaded well with a medical spurtle in a glass petri dish and divided into five parts (0.07 g) by using a balance. Each part was placed on wet filter paper in a plastic cup (120 ml) and administered to a 4th-stadium *B. mori* larva. The viral paste was completely consumed in 24 h at 25°C. Then the larva was supplied with 2.5 g Silkmate 2S. Larvae were reared until they died of viral infection or molted to the 5th stadium. The dead larvae were dissected and their degenerating tissue debris was observed with a microscope to detect viral polyhedra. Groups of five larvae were used to assay each viral dose. LD₅₀ was calculated by the limiting-dilution value and the number of polyhedra in the viral paste using the Spearman-Kärber method (Finney, 1964).

Dose effect of nikkomycin Z and flufenoxuron. Newly ecdysed 4th-stadium silkworm larvae were fed a diet (0.5 g/larva) containing nikkomycin Z or acetone-dissolved flufenoxuron. After 22 h of feeding, about 60–70% of the diet was consumed. Then the larvae were starved for 2 h and bioassayed for NPV susceptibility.

Observation of the peritrophic membrane. Ten newly ecdysed 4th-stadium silkworm larvae were fed a diet containing 10 ppm nikkomycin Z. After 22 h of feeding, the larvae were starved for 2 h and then dissected to expose their internal organs. Their alimentary canals were cut at a joint between the fore- and mid-guts. The peritrophic membrane was removed from their midguts with forceps and immersed in distilled water to observe with the naked eye.

Effect of nikkomycin Z on the development of the silkworm. Newly ecdysed 3rd- and 4th-stadium silkworm larvae were left with Silkmate 2S containing nikkomycin Z at 10 ppm. Then, they were transferred to a normal diet without nikkomycin Z and reared until they developed to the next stadium. The duration during which they were left with nikkomycin Z-containing diet is

shown in Table 3. Groups of 10 larvae each were used to assay each duration of the nikkomycin Z diet. The developmental stage was checked once a day. Days required to develop to the next stadium were analyzed by the Mann-Whitney *U*-test based on the duration of the nikkomycin Z diet.

RESULTS

Dose effect of nikkomycin Z and flufenoxuron

When the larvae were fed a diet containing nikkomycin Z at 1, 3, 10 and 100 ppm, the LD₅₀s were 2.8×10^5 – 2.8×10^6 , 1.1 – 7.0×10^1 , 0.7 – 1.1×10^0 and 0.4 – 1.1×10^0 polyhedra/larva, respectively (Table 1). The LD₅₀ of control larvae was 1.8 – 4.4×10^6 polyhedra/larva or more. The results indicated that the NPV-susceptibility was increased as the concentration of nikkomycin Z in the diet was increased in a range from 1 to 10 ppm. The effect of nikkomycin Z was assumed to be maximal at 10 ppm because the same degree of infectious enhancement was obtained at 10 and 100 ppm. No larvae that did not receive NPV polyhedra died of viral infection (data not shown).

Dose effect of flufenoxuron

When 4th-stadium silkworm larvae were fed diet containing acetone-dissolved flufenoxuron at 10 and 100 ppm, the LD₅₀s were 4.4 – 7.0×10^5 and 1.1×10^0 , respectively (Table 2). The LD₅₀ of control larvae was 1.8 – 2.8×10^6 . Acetone-dissolved flufenoxuron scarcely promoted NPV infection at 10 ppm. The results are similar to those obtained for 5th-stadium silkworm larvae by Arakawa (2002). Larvae that had not received NPV polyhedra did not die of viral infection (data not shown).

Peritrophic membrane of nikkomycin Z-treated larvae

Fourth stadium silkworm larvae that had ingested 10 ppm nikkomycin Z in the diet were dissected. The gut was filled with the diet ingested by the larvae, and the peritrophic membrane was not observed. In the control larvae that ingested a diet without nikkomycin Z, the tubular peritrophic membrane of surrounding the ingested diet was observed and could be removed from the gut with forceps and immersed in distilled water. The membrane of the control larvae was so tough that it retained its tubular form in the distilled water.

Table 1. Effect of nikkomycin Z on BmNPV infection in 4th-stadium *Bombyx mori* larvae^a

Concentration of nikkomycin Z (ppm)	Replicate	Number of larvae infected with NPV after ingestion of indicated number of polyhedra/larva										LD ₅₀ (polyhedra/ larva)
		2.22×10 ⁷	2.22×10 ⁶	2.22×10 ⁵	2.22×10 ⁴	2.22×10 ³	2.22×10 ²	2.22×10 ¹	2.22×10 ⁰	2.22×10 ⁻¹	2.22×10 ⁻²	
0	1	4	3	1	0	0	0	—	—	—	—	≥1.8×10 ⁶
	2	4	2	0	0	0	0	—	—	—	—	≥4.4×10 ⁶
	3	5	1	0	0	0	0	—	—	—	—	4.4×10 ⁶
1	1	5	2	0	0	0	0	—	—	—	—	2.8×10 ⁶
	2	4	4	2	2	0	0	—	—	—	—	2.8×10 ⁵
	3	5	2	2	0	0	0	—	—	—	—	1.1×10 ⁶
3	1	—	—	5	5	4	4	4	1	1	0	1.1×10 ¹
	2	—	—	5	4	3	4	3	2	0	0	4.4×10 ¹
	3	—	—	5	4	3	4	2	2	0	0	7.0×10 ¹
10	1	—	—	—	—	5	5	5	5	0	0	0.7×10 ⁰
	2	—	—	—	—	5	5	5	4	1	0	0.7×10 ⁰
	3	—	—	—	—	5	5	5	4	0	0	1.1×10 ⁰
100	1	—	—	—	—	5	5	5	3	1	0	1.1×10 ⁰
	2	—	—	—	—	5	5	5	5	1	0	0.4×10 ⁰
	3	—	—	—	—	5	5	5	4	0	0	1.1×10 ⁰

^a There were 5 larvae/test.
—: Not tested.

Table 2. Effect of flufenoxuron on BmNPV infection in 4th-stadium *Bombyx mori* larvae^a

Concentration of flufenoxuron (ppm)	Replicate	Number of larvae infected with NPV after ingestion of indicated number of polyhedra/larva										LD ₅₀ (polyhedra/ larva)
		2.22×10 ⁷	2.22×10 ⁶	2.22×10 ⁵	2.22×10 ⁴	2.22×10 ³	2.22×10 ²	2.22×10 ¹	2.22×10 ⁰	2.22×10 ⁻¹	2.22×10 ⁻²	
0	1	5	3	0	0	0	0	—	—	—	1.8×10 ⁶	
	2	5	2	0	0	0	0	—	—	—	2.8×10 ⁶	
10	1	5	5	0	0	0	0	—	—	—	7.0×10 ⁵	
	2	5	4	2	0	0	0	—	—	—	4.4×10 ⁵	
100	1	—	—	—	—	5	5	5	4	0	1.1×10 ⁰	
	2	—	—	—	—	5	5	5	4	0	1.1×10 ⁰	

^a There were 5 larvae/test.
—: Not tested.

Table 3. Developmental delay of *Bombyx mori* larvae ingesting 10 ppm nikkomycin Z

Larval stadium fed with nikkomycin Z	Duration of exposure to nikkomycin Z	No. of larvae used	No. of larvae molting to the next stadium	Days of the stadium (average $\pm$ SD)	Mann-Whitney <i>U</i> -test ^a
Third	0 h	10	10	4.3 $\pm$ 0.5	$p < 0.01$ $0.01 < p < 0.05$ $0.01 < p < 0.05$ $p > 0.05$
	24	10	10	5.7 $\pm$ 0.5	
	72	10	10	6.5 $\pm$ 0.5	
	120	10	10	7.1 $\pm$ 0.3	
	Throughout 3rd stadium	10	10	7.5 $\pm$ 0.5	
Fourth	0 h	10	10	5.3 $\pm$ 0.5	$p < 0.01$ $0.01 < p < 0.05$ $p > 0.05$
	24	10	10	6.5 $\pm$ 0.5	
	72	10	10	7.4 $\pm$ 1.0	
	120	10	10	7.8 $\pm$ 0.6	

^a Pairs of neighboring data were analyzed.

Effect of nikkomycin Z on the development of the silkworm

Nikkomycin Z did not prevent molting of the silkworm at 10 ppm. All the 3rd- and 4th-stadium larvae ingesting nikkomycin Z developed to the 4th- and 5th-stadium respectively (Table 3). The data were arranged in order of increasing exposure time of larvae to nikkomycin Z and the pairs of neighboring results were statistically analysed by a Mann-Whitney *U*-test. In most cases, the silkworm required a longer period to develop to the next stadium as the exposure time to nikkomycin Z increased. No significant difference was detected for the pairs of 120 h and throughout the 3rd-stadium and 72 h and 120 h of 4th-stadium larvae (Table 3).

DISCUSSION

It is assumed that nikkomycin Z interfered with chitin synthesis in the peritrophic membrane producing cells, resulting in the disruption of the membrane and the disappearance of its defensive function, leading to an increase in sensitivity to viral infection. Nikkomycin Z itself or its modified derivatives will serve as synergists of viral pesticides.

Many chemicals were reported to promote viral infection (see Goto, 1999). Among such chemicals, fluorescent brightener 28 has been most extensively studied (Shapiro and Robertson, 1992; Li and Otvos, 1999; Wang and Granados, 2000). Fluorescent brightener 28 has been used at a concentration of about 1% in the inocula of polyhedral suspension for satisfactory infectious enhancement. Arakawa (2002) showed that flufenoxuron,

an insect growth regulator, that inhibited insect molting promoted BmNPV infection only when this chemical was dissolved in acetone and administered to the silkworm *B. mori* perorally. Flufenoxuron that was incorporated into the diet as a powder form did not exert such an effect. Acetone-dissolved flufenoxuron was effective at concentrations higher than 50 ppm on the 5th-stadium silkworm larvae (Arakawa, 2002). In the present study, 10 ppm flufenoxuron scarcely promoted viral infection in the 4th-stadium silkworm. On the other hand, nikkomycin Z was so effective that the silkworm receiving this chemical at 3 ppm and 10 ppm was about 8.5×10^4 and 4.3×10^6 times more sensitive to BmNPV infection than control larvae based on LD₅₀, respectively. Thus, nikkomycin Z is the most likely candidate for a synergist of viral pesticides to date.

The peritrophic membrane was not observed in the gut of silkworms receiving 10 ppm nikkomycin Z. The peritrophic membrane is a barrier to microbes invading the insect perorally (Wang and Granados, 2000). BmNPV-free virions released from the polyhedra by the alkaline gut juice would easily gain access to the midgut cells of the silkworm treated by nikkomycin Z. Nikkomycin Z is an inhibitor of gut and epidermal chitin synthase (Cohen and Casida, 1980; Cohen, 1987; Decker et al., 1991). The peritrophic membrane of insects is a composite structure of chitin and numerous proteins, glycoproteins, and proteoglycans secreted by certain cells in the midgut epithelium (Richards and Richards, 1977; Hopkins and Harper, 2001). Thus, nikkomycin Z would promote viral infection by destroying the defensive function of the per-

itrophic membrane in the silkworm by inhibiting gut chitin synthase responsible for the membrane production. Chitin synthase inhibitors are candidates for IGRs. This study and a previous work (Arakawa, 2002) suggest that chemicals inhibiting chitin synthesis in insects should be considered candidates not only for IGRs but also for synergists of viral pesticides.

Nikkomycins are poor insect-control agents because the polar nature of these chemicals restricts their penetration through the hydrophobic insect epicuticle (Cohen, 1987). When ingested, these compounds are likely to be degraded inside the insect alimentary canal. Alternatively, their relatively high molecular weight prevents translocation across membranes of midgut cells (Cohen, 1987). In this study, nikkomycin Z did not inhibit the molting of the silkworm. On the other hand, Schluter (1982) reported that the Mexican bean beetle (*Epilachna varivestis*) larvae ingesting nikkomycin that was sprayed on a bean leaf died of failure in molting. The effect of nikkomycins on molting would vary among insect species. Whether or not the effect of nikkomycin Z to promote viral infection also varies among insect species remains open to future research.

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