Productivity and quality of polyhedral occlusion bodies of a nucleopolyhedrovirus harvested from *Spodoptera litura* (Lepidoptera: Noctuidae) larvae

Jun Takatsuka, Shohei Okuno, Takayoshi Ishii, Madoka Nakai and Yasuhisa Kunimi

Department of Bioregulation and Biointeraction, Graduate School of Agriculture, Tokyo University of Agriculture and Technology; Fuchu 183–8509, Japan

(Received 25 April 2006; Accepted 18 August 2006)

Abstract

We studied the yield and biological activity of polyhedral occlusion bodies (POBs) in a *Spodoptera litura* multcapsid nucleopolyhedrovirus (SpltMNPV) harvested from larvae of *S. litura* on different days postexposure to the virus (dpe) by dosing fifth-stadium larvae with the virus. The yield of POBs in live larvae at 5 and 7 dpe was approximately 10 and 40%, respectively, compared to that from cadavers 8 dpe onward. A similar trend was observed in biological activity. POBs in live larvae at 5 dpe were less infectious than those harvested after larval death. The infectivity of POBs in live larvae at 7 dpe was intermediate, but comparable to that of POBs harvested after larval death. The volume of POBs was related to differences in biological activity. The volume of POBs harvested at 5 dpe was significantly smaller than that harvested 7 dpe, which was significantly smaller than that harvested after larval death. We discuss the results with respect to the mass production of SpltMNPV in *S. litura* larvae.

Key words: Infectivity; microbial control; nucleopolyhedrovirus; production; *Spodoptera litura*

INTRODUCTION

The common cutworm *Spodoptera litura* (Fabricius) is a serious insect pest of many forage crops and vegetables in the subtropical and temperate regions of Asia and Oceania. A nucleopolyhedrovirus (NPV) that infects this insect is now being developed as a microbial control agent (Kunimi, 2002). Generally, NPVs are thought to be promising candidates for controlling insect pests because they have few negative effects on nontarget organisms and the environment (Hunter-Fujita et al., 1998; Takatsuka and Kunimi, 2003). However, certain factors limit the commercialization of NPVs for insect pest control agents, e.g., its high production cost.

Some virus species may be reproducible in insect cell culture, but the associated costs are relatively high (Hink, 1982). Therefore, all NPVs that have been developed as commercial products thus far have been produced in insect larvae. The procedure for the production of *S. litura* multcapsid nucleopolyhedrovirus (SpltMNPV) was investigated by Okada (1977), whereby SpltMNPV-killed larvae were collected using a special aspirator to harvest polyhedral occlusion bodies (POBs). However, Okada (1977) did not consider the harvest of POBs from live larvae. To obtain basic information to assess the possibility of harvesting viruses from live larvae for mass production, we studied both quantitative and qualitative aspects of SpltMNPV harvested at different numbers of days after exposure to the virus.

MATERIALS AND METHODS

Insect. *Spodoptera litura* was originally collected from a soybean field at the Field Science Center, Tokyo University of Agriculture and Technology, Tokyo, Japan, in 2000, and was continuously reared in our laboratory. Larvae were kept in plastic cages (30 cm × 22 cm × 6 cm) at 25°C with a...
16-h photoperiod until pupation, and were fed an artificial diet (Insecta; Nihon Nosan-Kogyo Co. Ltd., Yokohama, Japan). Individual adults were transferred into paper bags (8 cm × 15 cm × 20 cm) with a 10% crude sugar solution for feeding.

**Virus.** The SpltMNPV was originated from the diseased larvae of *S. littura* collected at Fukuyama, Hiroshima Prefecture, Japan (Okada, 1977; Takatsuka et al., 2003), and was isolated as an individual genotypic variant (SpltMNPV-C1) using the in vivo cloning method developed by Smith and Crook (1988). The original SpltMNPV was provided by the Genebank of the Ministry of Agriculture, Forestry and Fisheries. The restriction endonuclease profile of SpltMNPV-C1 was closely related to that of *S. littoralis* NPV-B type (Cherry and Summers, 1985). POBs of SpltMNPV-C1 were propagated in 70 fifth-stadium larvae of *S. littura* by feeding larvae POB-contaminated artificial diet (Insecta) at 25°C, purified by homogenization and density gradient centrifugation, suspended in 0.05 m sodium phosphate buffer (pH 7.7), and then counted using a Thoma hemocytometer (Kayagaki Irika Kogyo Co., Tokyo, Japan). Five counts per hemocytometer and three subsamples per suspension were measured to reduce counting and dilution errors. The suspensions were stored at 4°C until use.

**Larval mortality and productivity of NPV in larvae.** Insects beginning to molt out of the fourth stadium, determined by head-capsule slippage, were transferred into plastic cages without diet. The newly molted insects were collected after 16 to 20 h and then fed a diet for 24 h. Three groups of 50 one-day-old fifth-stadium larvae were transferred into plastic cages containing a diet treated with POBs at a rate of 5 × 10⁷ POBs per gram of diet. This rate maximizes the POB yield (Okada, 1977). As a control, the same number of larvae was fed a diet treated with the same solution, without POBs. After 24 h of feeding, larvae were individually transferred into 30-ml cups containing fresh artificial diet. The larvae were incubated at 25°C under a 16-h photoperiod. One POB-treated group and one control group were observed daily for mortality until pupation. Tissue smears prepared from dead larvae were examined for POBs under a phase contrast microscope. The other POB-treated groups were used for the enumeration of POBs in larvae. Sampling of larvae for this enumeration was conducted as follows. At 5, 7, 8, 9, and 10 days postexposure to POBs (dpe), 4–10 larvae were weighed, transferred individually to 10-ml tubes and stored at −20°C. Live larvae were sampled at 5 and 7 dpe, but both live and dead (virus-killed) larvae were sampled at 8 dpe. At 9 and 10 dpe, only dead larvae were sampled. Viral death generally caused liquefaction of the cadaver. Only cadavers that could be transferred whole from the cups were used. The sampled larvae were homogenized using Polytron PT 2100 (Kinematica AG, Lucerne, Switzerland) in sterilized distilled water and adjusted to 10 ml. Counting of POBs was performed using a Thoma hemocytometer. Experiments were replicated three times.

**Bioassay to assess the biological activity of harvested polyhedra.** The biological activity of POBs isolated from live larvae at 5 and 7 dpe and from dead larvae were examined using purified POBs from the homogenates that were used to enumerate POBs. Homogenates of virus-killed larvae at 8, 9, and 10 dpe were pooled and processed to purify POBs as described above. Second-stadium larvae were used for the bioassay experiments. Insects beginning to molt out of the first stadium, determined by head-capsule slippage, were individually transferred into a well on a 96-multiwell tissue culture plate. The newly molted insects were collected after 20–24 h for the bioassay.

Groups of 40 newly molted second-stadium larvae were transferred into 6-cm petri dishes; each dish contained eight artificial diet disks (8 mm in diameter, 1 mm in thickness). A POB suspension (20 μl) had been layered on the surface of the artificial diet disks. As a control, larvae were fed artificial diet disks treated with the same solution without POBs. After 24 h of feeding, 35 larvae were transferred into 30-ml cups containing fresh artificial diet. Five doses plus a control were tested. The experiments were replicated three times with 35 larvae per dose for each replication. The larvae were incubated at 25°C under a 16-h photoperiod and observed daily for mortality until pupation. Tissue smears prepared from dead larvae were examined for POBs under a phase contrast microscope.

**Estimation of polyhedra volumes.** POB suspensions purified from live and dead larvae were fixed on glass slides using formaldehyde. Slides were examined under a BX-51 microscope (Olym-
Quantitative and Qualitative Aspects of a NPV

RESULTS

Larval mortality

We examined the progression of *S. litura* larval mortality following exposure to the POB-treated diet (Fig. 1). Mortality was first observed at 6 dpe, at 0.9%. The average cumulative mortality was 8.4, 62.3, and 90.1% at 7, 8, and 9 dpe, respectively. All larvae were dead by 12 dpe.

Productivity of polyhedra

The POB yield, POB yield per unit larval weight, and larval weight of larvae sampled at different dpe are summarized in Table 1. The POB yield increased from 5 to 8 dpe. At 8 dpe, live larvae had fewer POBs than dead larvae, but the difference was not significant. There were no significant differences in POB yield among dead larvae collected at 8, 9, and 10 dpe. A similar trend was observed for POB yield per unit larval weight, which increased from 5 to 8 dpe. POB yield per unit larval weight in dead larvae was greater than that in live larvae, but the difference was not significant. The POB yield per unit larval weight in dead larvae was constant from 8 to 10 dpe. Larval weight tended to increase with time, but was not significant.

![Fig. 1. Progression of mortality after the exposure of *Spodoptera litura* fifth-stadium larvae to *S. litura* multicapsid nucleopolyhedrovirus. Error bars indicate standard error of the mean.](image)

Table 1. Yield of polyhedra per larva, yield of polyhedra per unit larval weight, and larval weight on different days post-exposure to virus

<table>
<thead>
<tr>
<th>Days post-exposure to virus</th>
<th>Number of larvae examined</th>
<th>Polyhedra were harvested from living or dead larvae</th>
<th>Log number of polyhedra&lt;sup&gt;a&lt;/sup&gt; (S.E.) per larva</th>
<th>Log number of polyhedra&lt;sup&gt;a&lt;/sup&gt; (S.E.) per mg larval weight</th>
<th>Weight of larvae in mg&lt;sup&gt;a&lt;/sup&gt; (S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>24</td>
<td>living</td>
<td>8.85 ± 0.14</td>
<td>5.94 ± 0.16</td>
<td>796.41 ± 63.45</td>
</tr>
<tr>
<td>7</td>
<td>25</td>
<td>living</td>
<td>9.23 ± 0.07</td>
<td>6.31 ± 0.07</td>
<td>834.20 ± 72.06</td>
</tr>
<tr>
<td>8</td>
<td>24</td>
<td>living</td>
<td>9.59 ± 0.04</td>
<td>6.67 ± 0.05</td>
<td>944.81 ± 81.48</td>
</tr>
<tr>
<td>8</td>
<td>24</td>
<td>living</td>
<td>9.72 ± 0.07</td>
<td>6.82 ± 0.04</td>
<td>836.53 ± 43.79</td>
</tr>
<tr>
<td>9</td>
<td>28</td>
<td>dead</td>
<td>9.78 ± 0.05</td>
<td>6.81 ± 0.06</td>
<td>965.36 ± 96.71</td>
</tr>
<tr>
<td>10</td>
<td>22</td>
<td>dead</td>
<td>9.77 ± 0.06</td>
<td>6.82 ± 0.06</td>
<td>938.44 ± 72.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Values followed by the same letter were not significantly different (*p*> 0.05), based on Tukey's HSD.
Biological activity of harvested polyhedra

Significant effects on mortality occurred among POB suspensions prepared from larvae sampled at different dpe ($\chi^2=13.43$, df=2, $p=0.0012$) and concentrations of POB suspensions ($\chi^2=284.16$, df=1, $p<0.0001$); however, the interaction was not significant ($\chi^2=0.28$, df=2, $p=0.8688$). POBs harvested from live larvae at 5 dpe were less infectious than those harvested from dead larvae at 8 to 10 dpe. The infectivity of POBs harvested from live larvae at 7 dpe was intermediate (Fig. 2). The 50% lethal concentration of POB suspensions prepared from live larvae sampled at 5 and 7 dpe, and from dead larvae were $1.74\times10^5$ POBs/ml (95% confidence interval [CI], 1.35×$10^5$–2.29×$10^5$ POBs/ml), 1.05×$10^5$ POBs/ml (95% CI, 8.02×$10^4$–1.39×$10^5$ POBs/ml), and 8.95×$10^4$ POBs/ml (95% CI, 6.34×$10^4$–1.17×$10^5$ POBs/ml), respectively.

Polyhedra volume

The distribution patterns of POB volumes were unimodal and positively skewed (Fig. 3). The mode tended to increase with increasing incubation period. POB volumes harvested at different dpe differed significantly ($F_{1,1396}=459.13$, $p<0.0001$). The POB volume harvested from live larvae at 5 dpe was significantly smaller than that harvested from live larvae at 7 dpe and from dead larvae (Tukey HSD, $p<0.01$). The POB volume harvested from live larvae at 7 dpe was also significantly smaller than that harvested from dead larvae (Tukey HSD, $p<0.01$). The average POB volume harvested from live larvae at 5 and 7 dpe and from dead larvae was 1.59 (standard error [SE], 0.046), 2.65 (SE, 0.044), and 3.63 (SE, 0.065) $\mu$m$^3$, respectively.

DISCUSSION

The timing of POB harvest played a critical role in determining the quality and productivity of POBs. Both the yield and infectivity of POBs harvested from NPV-killed larvae were higher than those from NPV-infected live larvae. Therefore, it may be best to harvest POBs after larval death. However, this involves the practical difficulty of harvesting ruptured and disintegrating larvae. Frequent observation and collection of larvae just after death over a period of several days should maximize the number of POBs to be harvested, but is quite laborious. Okada (1977) developed an appa-
ratus that could be used to aspirate and collect ruptured and disintegrating larvae. Collection of larvae using this apparatus diminished the labor and loss of POBs associated with difficulties in harvesting liquefying larvae. However, bacteria and fungi in NPV-infected larvae increase in number during the incubation period, reaching high numbers after larval death (Ignoffo and Shapiro, 1978; Smits, 1987; Grzywacz et al., 1998). Product analysis guidelines for microbial pest control agents proposed by the U.S. Environmental Protection Agency place a limit on the number of microbes allowed in the product. The guideline for microbial pesticides proposed by the Ministry of Agriculture, Forestry and Fisheries of Japan does not show this kind of the limit but requests to describe methods of preventing a biological alien substance into the products (the Ministry of Agriculture, Forestry and Fisheries of Japan, 1997). The removal of microbial contaminants from the product, however, is expensive (Shapiro, 1982) and sometimes found to be difficult (Grzywacz et al., 1997). Microbial contamination in NPV-infected live larvae would be lower than that in NPV-killed larvae. In the study on S. littoralis NPV production, Grzywacz et al. (1998) described that the virus product from live larvae was only contained with acceptable level of microbes. In this sense, harvesting virus from live larvae is probably advantageous over doing from NPV-killed larvae.

Another subject to be considered is a rearing condition, that is to say, rearing NPV-infected larvae in aggregate or individually. From economic standpoint, it is less costly and more efficient to rear the larvae in aggregate (Shapiro, 1982), because it reduces labor, therefore, reduces labor-associated costs that share approximately 70% of total production costs of viral insecticides (Okada, 1977; Kunimi, 1986). However, it is well known that S. litura larvae cannibalize, which precluded rearing of NPV-infected larvae in aggregate for mass production of the virus (Okada, 1977). This behavior is most frequent among S. litura larvae when vital and moribund larvae are present together. In our study, mortality was not synchronized, instead occurred from 7 to 12 dpe, during which both infected but yet to be vital and moribund larvae were present together. Rearing NPV-infected larvae in aggregate and harvesting virus from NPV-killed larvae cause substantial losses of NPV-killed larvae to be recovered, in a worst case only about 10% of NPV-killed larvae were recovered (Takatsu et al., unpublished). However, until 7 dpe when mortality occurred only at low rate, the loss due to cannibalism was small and almost NPV-infected larvae can be recovered even if the larvae were reared in aggregate (Takatsu et al., unpublished). Although approximately 40% fewer POBs were harvested from NPV-infected live larva at 7 dpe than from NPV-killed larva, their infectivity did not differ. Thus, rearing NPV-infected larvae in aggregate and harvesting the virus early during the incubation period, when larvae are still alive, may be the best practical strategy in commercial mass production of the virus.

Differences in the biological activity of POBs harvested at different dpe were likely correlated with the size of the POBs. POBs were used as the dosage unit, but smaller POBs may result in a lower dose of virions; POB volume is positively correlated with the number of virions enclosed in the POB (Mazzone and McCarthy, 1981; Allaway, 1983). Ignoffo and Shapiro (1978) observed seven to nine times higher infectivity of POB preparations from virus-killed larvae than from live larvae in the NPV–Heliothis zea system. Up to 12 times higher infectivity of POBs from virus-killed larvae was detected in the NPV–Lymantria dispar system (Shapiro and Bell, 1981). In the MNPV–Spodoptera exigua system, Smits (1987) detected slightly higher activity of POBs from virus-killed larvae at lower concentrations. Smits (1987) also observed that POB suspensions prepared from live larvae contained many small POBs.

The yield per unit weight, which indicates the rate of conversion of insect to virus, increased from 7 to 8 dpe and onward, without a significant increase in larval weight. This implies that POBs could increase in number without any dietary input. Therefore, to improve the amount of POBs to be harvested, the incubation of SpplMNPV-infected live larvae from 7 dpe onward without a diet may be a good strategy. Smits (1987) proposed this approach in producing S. exigua MNPV. Studies of the production of S. littoralis NPV in S. littoralis larvae have shown that this approach gives an improved POB yield without the unacceptable increase in bacterial contaminants, when the incubation is conducted at a relatively low temperature (e.g., 14°C; Grzywacz et al., 1998).
The selection of a more active viral strain or the use of substances that enhance viral activity would also benefit this system. Unfortunately, we have not yet been successful in identifying a strain that has higher biological activity than SpltMNPV-C1 (Takatsuka et al., 2003, unpublished data). We have, however, found that some substances enhance the activity of NPVs that infect S. littura. For example, some optical brighteners enhanced the viral activity of SpltMNPV up to 1700 times in fourth-stadium S. littura larvae (Okuno et al., 2003). Thus, the use of the substances that enhance viral activity would further reduce the costs for controlling S. littura by the viral insecticides.

ACKNOWLEDGEMENTS

We thank N. Onigahara for technical assistance. This work was supported by a grant for the research and development program for new bio-industry initiatives (Consortium 3: the development of new microbial biopesticides) from the Bio-oriented Technology Research Advancement Institution, Japan.

REFERENCES


