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BIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF *SPODOPTERA EXIGUA* NUCLEAR POLYHEDROSIS VIRUS (SeMNPV) (*BACULOVIRIDAE*) STORED FOR A LONG TIME AT DIFFERENT FORMULATIONS

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Abstract: Baculoviruses are widely used as bioagents for controlling insect populations. Although they are successfully replicated in cell cultures, still the production in laboratory reared larvae is the cheapest way for large-scale production of viral agents. Commercial products are prepared as liquid suspensions and as dried powders.
We investigated the stability of two formulations of *S. exigua* nuclear polyhedrosis virus (SeMNPV) stored at +4°C for over twenty years as a powder (prepared by aceton precipitation) and in a suspension. The biological activity and biochemical properties of these formulations were examined. Viral biological activity of the suspension was 1000 times better than the activity of powdered virus. Aceton precipitation method caused the significant loss of virus activity. Electrophoretic analysis of proteins showed degradation of polyhedrin peptide. There was also partial DNA degradation. These changes may lead to decreased bioactivity of powdered SeMNPV virus.

Key words: *Spodoptera exigua*, nuclear polyhedrosis virus, storage stability

INTRODUCTION

Commercial products based on baculoviruses are produced on a large scale in laboratory-reared larvae, infected by virus added to their food. Dead larvae are then collected, and the virus inclusion bodies are liberated from their tissues and prepared as suspension or dry powder (Shapiro 1982; Smits 1987). The method used to obtain a concentrated powder formulation is coprecipitation of a suspension of occlusion bodies in lactose with acetone (Dulmage et al. 1970). The problem of storing the virus for further use has not been deeply examined. The main interest was concentrated on increasing baculovirus infectivity and speed of action. It has

been reported that virus prepared by acetone precipitation loss activity in storage at high temperatures (35°C) (Hunter et al. 1977; McGaughey 1975; Ignoffo and Shapiro 1978).

The investigations presented the biological and biochemical characteristics of the same isolate of *S. exigua* nuclear polyhedrosis virus (SeMNPV) stored at +4°C for many years in a suspension and as a powder. The aim was to evaluate which form of storage was more useful, in which the virus stays infectious for longer time. This is essential in preparation of bioinsecticides consisting of baculoviruses.

MATERIAL AND METHODS

Viruses

The experiments were performed using two probes of *S. exigua* nuclear polyhedrosis virus (SeMNPV) stored in +4°C. One probe (stored 28 years) was purificated water suspension (SeS) and second (stored 26 years) was prepared as a powder using lactose and acetone (SeP) (Dulmage et al. 1970). Nine years old isolate served as a control (SeC).

Insects

L_4 larvae of the beet armyworm *S. exigua* reared on a semisynthetic diet (Poitout and Bues 1970) at 25°C, 70% relative humidity and 16:8 hr (L:D) photoperiod were tested.

Propagation of viruses and bioassays

Powdered virus was dissolved in distilled water to remove lactose present in the powder. All suspensions (SeS, SeP, SeC) were centrifuged at 6800 rpm for 15 minutes. The pellets were resuspended in the sterile distilled water and polyhedra from three suspensions were counted. The larvae were infected with five doses of polyhedra (10^5–10^{11} polyhedra/larva). Insects were infected *per os* with polyhedral bodies by contaminated diet. Each larva (30 larvae for virus concentration) was placed in separate glass vial and given a small piece of diet with 20 μl virus solution. For further tests only larvae which ingested the whole portion of diet during 24 hrs were used. After that larvae were feeding normally. Mortality was recorded every day until the larvae died or pupated. Died larvae were collected and kept at –20°C for further analysis.

Bioactivity of SeMNPV was analysed by probit methods LD_{50} value – according to Finney's method (Lipa and Śliżyński 1973) and LT_{50} value – according to Lithchfield and Wilcoxon method (Śliżyński and Lipa 1973).

Isolation of viral DNA

Viral DNA was isolated from 10^{10} occlusion bodies (OBs) from each SeS, SeP and SeC. Polyhedra were treated with 0.3 M Na_2CO_3 (sodium carbonate) pH≈11 for about 30 minutes at 37°C to dissolve the polyhedrin. Undissolved OBs were separated by low speed centrifugation and the supernatant was collected and incubated overnight at 55°C with proteinase K (100 μl/ml) with addition of SDS to concentration of 0.1%. After short centrifugation, the supernatant was treated with phenol:chloroform solution a few times and, subsequently, one time with chloroform

only. Then the DNA was extracted with 2.5 volume of 96% ethanol and 0.3 volume of 7.5 M ammonium acetate at –20°C. Next it was centrifuged, washed with 75% ethanol, air-dried and dissolved in small amount of ddH_2O.

Polymerase chain reaction

Two sets of primers were used according to fragment sequences of SeMNPV polyhedrin and p10 genes (Van Strien et al. 1992; Zuidema et al. 1993). The sequence of SeMNPV polyhedrin region upper primer was 5'AAAAACGCCAAACGCAAGGAGCAT3' and the lower primer was 5'TAGTCGGGATCGCAACGGAGAGC3' . The sequence of SeMNPV p10 region upper primer was 5'TACTTTTGATCCGAGCCGACATTA3' and the lower primer was 5'TTTGGCCGACAGCTCTGAAGTATC 3'. All primers were designed using the computer program DNAStar. Primers were synthesized by TIPMOLBIOL (Poland).

Each PCR reaction mixture consisted of: 0.1 μg DNA sample, 1U Taq polymerase (Sigma), 10 × reaction buffer, 100 μM dNTP and 0.5 μM of each primer. The final volume of one sample was 10 ml. The cycling parameters were as follows: 2 minutes 95°C (denaturation), 1 minute 54°C (annealing) and 1 minute 72°C (elongation). The number of cycles was 30. The polymerase chain reactions were performed in the thermocycler UNO-Thermoblock (Biometra, Germany) with heated lid. No mineral oil was added.

PCR products were separated on 2% agarose gel with ethidium bromide by 30 minutes electrophoresis at 100 mA and visualised at transilluminator UV. The molecular weights of PCR products were estimated by comparing to DNA weight markers (Roche).

Polypeptide analysis

Polyhedra of all types were solubilized in a sample buffer (25 mM Tris-Cl pH 7.5, 10 mM sodium metabisulfite, 1 mM PMSF, 10 mM DTT, 1 mM EDTA and 20% glycerol) by boiling for 3 minutes. Approximately 10 mg of protein were present in each line. The protein concentration was estimated by Lowry method (Lowry et al. 1951). 5% bromophenol blue was added, and electrophoresis was carried out in 12% polyacrylamide gel at 20 mA for 6–8 h. Gel was stained with a solution of 25% methanol, 10% acetic acid and 0.12% Coomassie blue R250 for about 1 h and then destained with 20% methanol and 10% acetic acid. The molecular weights were estimated by comparing to standard low weight marker (Merck).

RESULTS AND DISCUSSION

Polyhedrin is the most representative protein for nuclear polyhedrosis viruses. It protects virions against all unfavourable environmental factors. In presented work, we examined viruses from the same strain stored in two different formulations: in suspension and as a powder. Analysis of proteins using SDS-PAGE showed evident degradation of peptides in viruses stored as a powder (Fig. 1). In other lines a polypeptide band with an apparent molecular weight of about 30 kDa represents polyhedrin. Lower, also significant band may represent a major nucleocapsid protein of 28–29 kDa. Other bands correspond to other virions proteins, which are the

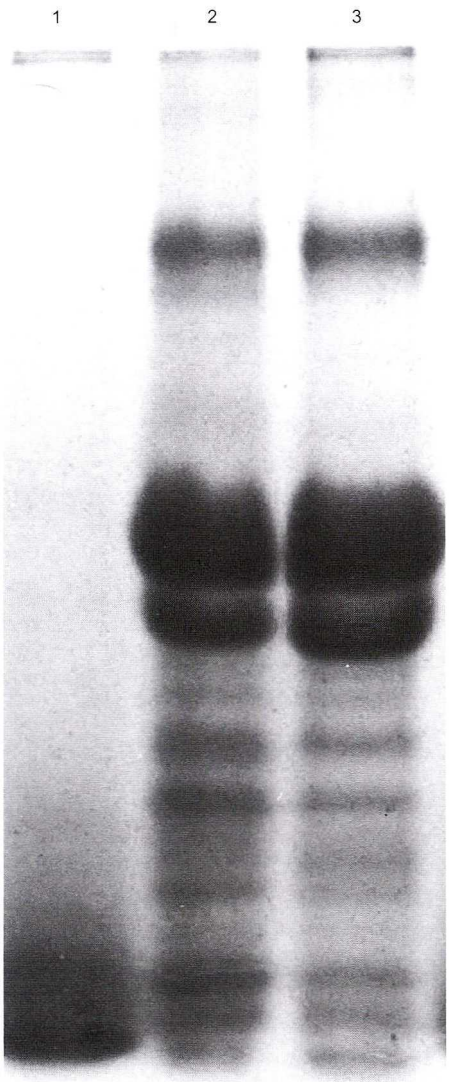

Fig. 1. Electrophoresis of the polyhedrins of the SeMNPV stored in the powder (line 1), in the suspension (line 2) and control virus (line 3) in the 12% SDS-PAGE gel

same for other viruses. In the case of powdered viruses storing caused degradation of viral proteins, which may lead to the conclusion that the manner of storage influences viral infectivity. Although powdering is considered as the most convenient way of preparing viral and other bioagents, there is a possibility that Dulmage method involving acetone causes some defects in polyhedrin structure. Uncovered virions are therefore exposed to all environmental factors. Viral genome may become destroyed and that may cause the virus deactivation.

Baculovirus genome is rather large and therefore difficult to isolate. Difficulties in isolating the whole DNA of powdered viruses may indicate its partial degradation. Those problems, together with relatively small approachable amounts, resulted in the idea of using PCR method for DNA analysis. Polymerase chain reaction is considered as one of the most sensitive methods of DNA detection. Two sets of primers were designed in order to amplify regions of polyhedrin and p10 genes. We obtained products for polyhedrin regions for all viruses (Fig. 2). With other combinations (p10 set of primers, p10 forward and polyhedrin forward primer) there were products only in the case of viruses stored in the suspension and control virus (Figs. 3, 4). Examined probes are the same strain of *S. exigua* MNPV, and differ only in the manner of storage. Differences in the PCR products may be the evidence for partial DNA degradation when stored as a powder. Viral DNA is responsible for the virulence. Proper virus replication and propagation of viral progeny depends on viral genes. Any disorder in the genome may lead to decreased activity. That was confirmed by bioassays for infectivity: the bioactivity of viruses stored as a powder was about 1000 times lower than the activity of viruses stored in the suspension and about 10^6 times lower than

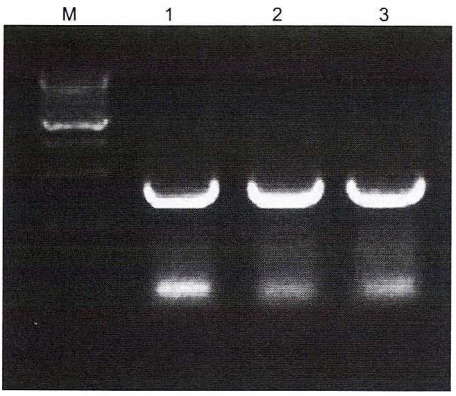

Fig. 2. Electrophoresis of the polyhedrin fragment gene PCR products for SeMNPV stored in the powder (line 1), in the suspension (line 2) and control virus (line 3)

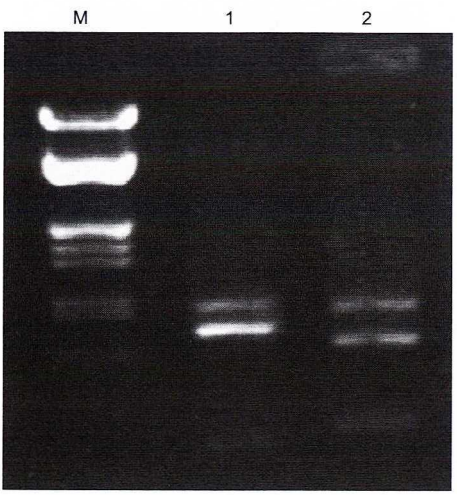

Fig. 4. Electrophoresis of the PCR products with p10 gene forward primer (line 1) and polyhedrin gene forward primer (line 2) for SeMNPV stored in the suspension

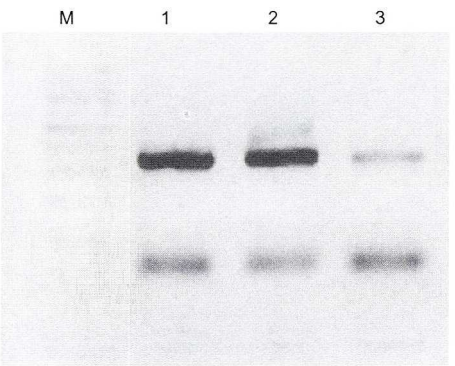

Fig. 3. Electrophoresis of the p10 fragment gene PCR products for SeMNPV control virus (line 1), stored in the suspension (line 2) and in the powder (line 3)

the control (Tabs. 1, 2). The LD_{50} and LT_{50} values of SeS, SeP and SeC were 5.3×10^{10}; 9.1×10^{13} and 1×10^{7} polyhedra/larva and 14.1; 18.0 and 5.7 days respectively. The activity of viruses stored for such a long time (28 and 26 years) in these formulations has not been examined yet. The influence of different storage temperatures has already been reported. Tamez-Guerra et al (2002) compared storage stability of *Anagrapha falcifera* nuclear polyhedrosis virus (AfMNPV) in spray-dried formulations to homogenate, stored at room temperature and refrigerated for 1 year. Unformulated virus stock maintained insecticidal activity better during storage than dried formulations. Unformulated virus, which is essentially a suspension of virus occlusion bodies in homogenized insect cadavers, did not lose activity when was stored at refrigerated or room temperature. They report that stability of AfMNPV insecticidal activity during storage as dry formulations is related to the general composition of the formulation. In the Dulmage method the use of acetone may negatively affect viral stability by disrupting polyhedrin which decreases biological activity, which was also reported by Ignoffo and Shapiro (1978).

Table 1. Values of median lethal doses (LD_{50}) for *S. exigua* larvae infected with SeMNPV stored in different formulations

Virus formulation	Age of virus probe (years)	Number of larvae tested	Virus concentration (polyhedra/larva)	LD_{50} values (polyhedra/larva)	Fiducial limits (95%)	Regression line y = bx + a
Suspension	28	150	$1\times10^{7} - 1\times10^{11}$	5.3×10^{11}	$3.6 \times 10^{9} - 7.8 \times 10^{13}$	y = 0.210116 x – 2.535972
Powder	26	150	$1\times10^{7} - 1\times10^{11}$	9.0×10^{13}	$1.7 \times 10^{9} - 4.9 \times 10^{18}$	y = 0.175253 x – 2.554003
Control	9	150	$1\times10^{5} - 1\times10^{9}$	1.0×10^{7}	$6.1 \times 10^{6} - 4.8 \times 10^{7}$	y = 0.493767 x – 1.427047

Table 2. Values of median lethal times (LT_{50}) for *S. exigua* larvae infected with SeMNPV stored in different formulations

Virus formulation	Age of virus probe (years)	Number of larvae tested	Virus concentration (polyhedra/larvae)	LT_{50} values (days)	Fiducial limits (95%)	Regression line y = bx + a
Suspension	28	150	$1\times10^{7} - 1\times10^{11}$	14.1	11.1 – 1.9	y = 3.837503 x +0.586548
Powder	26	150	$1\times10^{7} - 1\times10^{11}$	18.0	10.0 – 32.2	y = 4.612454 x +0.793559
Control	9	150	$1\times10^{5} - 1\times10^{9}$	5.7	4.8 – 6.8	y = 3.497346 x + 2.344617

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POLISH SUMMARY

BIOLOGICZNA I BIOCHEMICZNA CHARAKTERYSTYKA WIRUSA POLIEDROZY JĄDROWEJ SPODOPTERA EXIGUA (SeMNPV) (*BACULOVIRIDAE*) PRZECHOWYWANEGO PRZEZ DŁUGI OKRES W RÓŻNYCH FORMULACJACH

Zbadano aktywność biologiczną i właściwości biochemiczne wirusa poliedrozy jądrowej światłołówki naziemnicy *Spodoptera exigua* (SeMNPV) przechowywanego w formie zawiesiny (28 lat) oraz proszku przygotowanego metodą Dulmage'a (26 lat) w temperaturze +4°C. Wykazano znaczne obniżenie aktywności biologicznej wirusa w formulacji sproszkowanej. Była ona około tysiąckrotnie niższa w porównaniu z zawiesiną oraz około 10^6 razy niższa w porównaniu z kontrolą. Metoda laktozowo-acetonowej precypitacji zastosowana do przygotowania proszku wirusowego wprawdzie minimalizowała stopień zanieczyszczenia mikrobiologicznego wpływała jednak niekorzystnie na poliedryczne białko (poliedryna) bakulowirusa. Wykazała to elektroforetyczna analiza poliedryny oraz analiza DNA. Należy sądzić, że jest to jedna z istotnych przyczyn spadku wirulencji wirusa.