Journal of Biotechnology 259 (2017) 19–25

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

# *In vitro* production of *Spodoptera exigua* multiple nucleopolyhedrovirus with enhanced insecticidal activity using a genotypically defined virus inoculum

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ARTICLE INFO

*Keywords:*
*Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV)
Insecticides
A genotypically defined virus inoculum
Defective virus
Insect cell culture
*in vitro* production

ABSTRACT

Defective virus accumulations during baculovirus passages in insect cell culture are impediments to large scale baculovirus production. A genotypically defined virus inoculum comprises of stable genotypes was proposed for production of a Thailand isolated SeMNPV in Se-UCR1 insect cells. Targeted genotypes were from wild-type SeMNPV containing naturally mixed genotypes. Plaque assays, PCR screening and *Xba*I restriction analysis were employed for genotype purification, genotype selection and genome analysis, respectively. A selective marker was *pif2* encoded *per os* infection factor which predominantly deleted, along with the adjacent *pif1*, in defective viruses. A purified, genetically stable *pif2*+ (and *pif1*+) genotype, namely SeTh*pif2*+, was the first tryout. SeTh*pif2*+ occlusion bodies (OBs) possessed insecticidal activity but at lower level than the wild-type. When the SeTh*pif2*+ was co-infected with another purified, genetically stable *pif1*- (and *pif2*-) genotype, SeTh*pif2*-, at ratio of 3:1, respectively, mixed genotypes OBs had 2.8 times greater insecticidal activity than the SeTh*pif2*+ alone. Dilution of deleterious PIF1 of SeTh*pif2*+ by the *pif1* deletion genotypes, SeTh*pif2*-, was the key for this enhanced activity. A promising approach was described for SeMNPV production *in vitro* using the virus inoculum whose genotypes compositions were designed to mimic virus interactions in the wild-type, to generate *per oral* infective baculovirus.

## 1. Introduction

*Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV), a member of group II NPVs, is highly pathogenic and monospecific to beet armyworm (*Spodoptera exigua*) (Smits and Vlak, 1988). The SeMNPV has been isolated from different regions throughout the world (Vlak et al., 1981; Gelernter and Federici, 1986; Hara et al., 1995; Muñoz et al., 1998). Because of its narrow host range and being relatively virulent to its hosts, the SeMNPV occlusion bodies (OBs) are used as bio-pesticides. Although high yields of effective SeMNPV OBs are produced from infected larvae, microbial contaminations are often found and affect the physical stability and insecticidal activities of formulated products. Technology based on the viral propagation in insect cell cultures, in which the production process is standardized, environmental controlled and scalable, could be considered as an alternative, providing that several existing restrictions are overcome.

NPV has one double-stranded circular DNA genome packed in a protein capsid which in turn is enveloped by a lipoprotein membrane to form a virion. NPVs are found as large proteinaceous occlusion bodies (OBs) with one or more virion embedded. OBs are dissolved in the alkaline environment of the host mid-gut when consumed by susceptible insect larvae and their occlusion derived virions (ODVs) released to infect columnar epithelial cells of the larvae mid-gut. This primary infection is enabled only with the presence of viral encoded proteins i.e. P74 (Kuzio et al., 1989; Yao et al., 2004; Zhou et al., 2005), PIF1 (Kikhno et al., 2002), PIF2 (Pijlman et al., 2003), PIF3 (Ohkawa et al., 2005) and PIF4 (Ac150) (Lapointe et al., 2004; Zhang et al., 2005). In the early stages of infection, individual nucleocapsid is produced and then buds out of the infected insect cell as a budded virus (BV). Each cell within the insect is infected by an average of four to five BVs (Bull et al., 2001). Later stages of infection, nucleocapsids that remain in the nucleus, are enveloped in a polyhedron crystal protein to form virions that are occluded in a strong protein matrix as occlusion bodies (OBs) and then released from the dead larvae.

The SeMNPV genome is approximately 130 kbp (Caballero et al., 1992) and their complete sequence and gene organization have been

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http://dx.doi.org/10.1016/j.jbiotec.2017.08.001
Received 5 February 2017; Received in revised form 10 July 2017; Accepted 1 August 2017
Available online 02 August 2017

reported (IJkel et al., 1999). The SeMNPV naturally survives as mixed populations, comprises a number of different genotypes including naturally occurring deletion mutants (Lee and Miller, 1978; Maruniak et al., 1984; Smith and Crook, 1988; Muñoz et al., 1998). Genome deletion in SeMNPV wild-type isolates predominantly occurs within a single deletion of up to 25 kbp, spanning open reading frames (ORFs) 14–41 (Heldens et al., 1996; Muñoz et al., 1998; IJkel et al., 1999), containing some important genes for insect infection such as *pif1* and its neighboring gene *pif2*, that encode *per os* infection factors, and genes responsible for larvae liquefaction i.e. the *cathepsin* (ORF16) and *chitinase* (ORF 19) (Hawtin et al., 1997). This deletion mutant has become oral infection defective towards the insect larvae. When the defective genotype was co-infected with a complete genotype into an insect larvae, they survive by obtaining gene products of complete genotypes in co-infected cells. Although *pif1* is an essential gene needed for insect larvae infection, PIF1 appears to be deleterious if present in amounts higher than required. The deletion mutants that effectively dilute the intercellular pool of PIF1 are thus necessary and beneficial to the entire virus population. This mutual interaction result in increasing potency of the NPVs virus population (López-Ferber et al., 2003; Clavijo et al., 2009; Simón et al., 2013).

The SeMNPV budded virus can replicate in susceptible cell lines derived from *S. exigua* such as Se-UCR1 (Gelernter and Federici, 1986; Hara et al., 1993, 1994) and Se301 (Hara et al., 1995). SeMNPV infection into Se-UCR1 cell line produced highly infectious budded virus in cultured cells but their OBs was reported to lack infectivity for insects due to deletion in the SeMNPV genome (Heldens et al., 1996). These deleted genotypes rapidly accumulate in the SeMNPV virus population, become predominant after prolonged passaging in cell culture and eventually being ineffective bio-pesticides (Kool et al., 1991).

A procedure for preparation of effective baculovirus inoculum using specific genotypes was proposed to improve insecticidal efficiency of SeMNPV produced in insect cell culture. Methods include isolation of viruses using conventional plaque assay. Isolated plaques were then selected based on the presence of genes required for oral activity in insect using PCR analysis. We focused on detection of two genes encode *per os* infection factors, *pif1* and *pif2*, since they locate in the region often deleted in the defective NPV virus. Several rounds of plaque assays were performed to remove defective viruses or undesirable genotypes and allowed selection of only genetically stable viruses which are resistant to the genetically changes by *in vitro* passaging. Since the insecticidal activity of *pif1* and *pif2* containing viruses could be enhanced by dilution of PIF1, the *pif1* and *pif2* deletion genotype was also isolated and produced in insect cell culture to be used in the genotypically defined virus inoculum.

## 2. Materials and methods

### 2.1. Insect larvae

*Spodoptera exigua* larvae were obtained from the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. They were maintained under constant environmental conditions i.e. at 27 °C, 70% humidity, and a photoperiod of 16 h light and 8 h dark, and reared on an artificial diet.

### 2.2. Cell lines and viruses

*Spodoptera exigua* insect cell line, Se-UCR1, was used in this study with permission from Prof. Brian A. Federici, University of California, Riverside, USA. Cells were maintained in TNM-FH insect medium supplemented with 10% fetal bovine serum (FBS) and 0.25% Pluronic® acid at 27°C. SeMNPV was originally collected from infected *S. exigua* from central Thailand and was propagated in *S. exigua* larvae. Haemolymph was obtained from infected larvae fed with SeMNPV occlusion bodies (OBs) contaminated diet, filtered sterile, then added into an exponentially growing Se-UCR1 cell culture and incubated at 27°C. Three days post infection, budded viruses (passage no.1) in culture medium were harvested and purified by plaque assay (O'Reilly et al., 1994). Plaques were obtained and individually propagated in Se-UCR1 cells cultured in 24 well plates at 27 °C for 3 days. Budded viruses in culture media from each well were collected and their titers estimated by end point dilution method (Lynn, 1992). Infected cells containing OBs were collected and extracted by addition of 300 µl of 0.1% SDS, mixed vigorously and the mixtures were centrifuged at 6,000 rpm for 15 s to sediment cell debris. Then supernatant containing OBs was centrifuged at 10,000 rpm for 20–30 s. The OBs pellet was resuspended in 400 µl distilled water and their numbers were counted under a light microscope using an improved Neubauer hemocytometer.

### 2.3. Restriction endonuclease analysis of viral genomic DNA

Viral genomic DNA was prepared from occlusion bodies (OBs) using a modified method of Muñoz et al. (1997). The DNA was released from $10^9$ OBs in 300 µl sterile water. One-third volume of polyhedra dissolving solution (0.5 M $Na_2CO_3$, 0.5 M NaCl and 0.03 M EDTA, pH 10.5 and 500 µg/ml proteinase K) were added and the mixture was incubated at 50°C for 2.5 h. The viral genomic DNA in the solution was further extracted with Phenol: Chloroform: Isoamyl Alcohol (25:24:1) followed by ethanol precipitation. Three µg of viral genomic DNA was digested with 10 U of *Xba*I restriction enzyme at 37°C overnight. Electrophoresis of *Xba*I digested DNA was performed using 1% agarose gels. *Xba*I fragment sizes on gel were determined by Fragment Analyzer™ Auto CE System Program.

### 2.4. Rapid identification of virus genotype by PCR analysis

Budded viruses in culture medium from plaque infected cells were mixed with an equal volume of 35% PEG at 4°C overnight and centrifuged at 15,000 rpm for 1 h. Viral genomic DNA from the pellet was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) saturated with TE followed by ethanol precipitation. Rapid genotype determination was performed using PCR detection of specific genes, *pif2* (nt 35146–36387). Primers were designed in accordance with the complete genomic sequence of Se-US1A virus (Gene Bank accession no NC002169) (IJkel et. al., 1999) where its 3′ is overlapped with *pif1* promoter. *Pif2* forward and reverse primers were; 5′-CTGCAAT TAAAAACGGGAGAAC-3′ and 5′-TTGTATATGACGGCGATCAAGA −3′, respectively. *Chi* forward and reverse primers were; 5′-TGTTTGCGCTCTTAACGACA-3′ and 5′-ATCGGAGCAAATGAGGACAA-3′, respectively. Additional primers specific to the *p10* of SeMNPV were included for *p10* detection as a control.

### 2.5. Virus isolation by plaque assay

Specific SeMNPV genotypes in infected larvae hemolymph were isolated by plaque assays (O'Reilly et al., 1994). In the first round, seventy two plaques were obtained and propagated in Se-UCR1 cells cultured in 24-well plates. Ten out of seventy two samples in infected cell culture medium were randomly selected and their budded viruses were precipitated with 35%PEG. The viral DNA was extracted, precipitated and subjected to PCR analysis for *pif2* detection. Two groups were identified; a *pif2* positive group and a *pif2* negative group. A sample from each group namely SeTh*pif2*+ and SeTh*pif2*-, respectively, was randomly chosen for the next round of plaque purification. Serial plaque purifications were carried out until all plaque samples from each group were either *pif2*+ or *pif2*-. Each isolated viruses, SeTh*pif2*+ or SeTh*pif2*- was propagated and they were titer determined by end point dilution assay. *Xba*I REN analysis of viral genomic DNA and insect larvae bioassay of OBs produced from infected Se-UCR1 cells were performed for genotype and phenotype confirmation.