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*A. pernyi***

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Efficiency of *Antheraea pernyi* Nucleopolyhedrovirus-Mediated Protein Production in both an Established Cell Line and Diapausing Pupae of *A. pernyi*

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Abstract Efficiency of recombinant protein production in a novel baculovirus expression vector (BEV) system established by combining an established cell line (AnPe) and diapausing pupae of *Antheraea pernyi* with a baculovirus *A. pernyi* NPV (AnpeNPV) was compared with other BEV systems and evaluated. *In vitro* production using AnpeNPV/AnPe cell systems showed similar maximum β -galactosidase activity to the AcNPV/Sf9 and HycuNPV/SpIm cell systems, 5 times more than the BmNPV/BmN4 cell system and one-fifth of the AcNPV/High5 cell system. *In vivo* production using AnpeNPV/*A. pernyi* diapausing pupa system showed the maximum β -galactosidase activity similar (male pupa) to or significantly higher (female pupa) than BmNPV/*Bombyx mori* larva system. The results suggested that the AnpeNPV vector system using *A. pernyi* diapausing pupae has a potential to become more practical protein production system than *B. mori* larvae.

Key words: *Antheraea pernyi*, NPV, β -galactosidase, diapausing pupa, baculovirus expression vector, insect cell

Introduction

Since the baculovirus expression vector (BEV) systems using *Autographa californica* nucleopolyhedrovirus (AcNPV) and *Bombyx mori* NPV (BmNPV) were established (Smith *et al.*, 1983; Maeda *et al.*, 1985), they have been used for the high level expression of a wide variety of heterologous genes, especially higher eukaryotic genes which were difficult and/or problematic to express by the conventional bacterial expression system, and in many cases recombinant proteins obtained by BEV systems are structurally and functionally similar to their authentic counterparts (Luckow and Summers, 1988).

There are two possible ways for the

practical production of recombinant proteins in BEV systems, one of them is *in vitro* production using insect cell lines and the other is *in vivo* production using insect larvae. In general, recombinant proteins produced *in vitro* are relatively homogeneous and can be easily purified from culture medium and/or infected cells. However, costs of media and equipments for the cell culture are too expensive to hamper the economical scale-up. In contrast, large amounts of recombinant proteins can be produced at lower costs by the *in vivo* system using *B. mori* larvae which have been completely domesticated and whose rearing technology has been quite sophisticated in the long history of the sericulture. In addition, the tissue-specific and complicated post-translational modifications, which are not observed in

cultured insect cells, can be occurred in various differentiated cells in larvae, although resulting recombinant proteins become more heterogeneous than *in vitro* production. The major drawback of *in vivo* production is difficulty in the purification of recombinant proteins from large amounts of larval proteins as well as other materials. *In vivo* production also requires laborious tasks of larval manipulation, such as recombinant virus inoculation and hemolymph and/or tissue collection.

For *in vitro* production system using the AcNPV, many technical improvements have been added and several user-friendly kits are now available from various companies. A few kits of *in vivo* production using *B. mori* larvae are also available, however, it is very difficult for any laboratories without insect rearing facility to introduce these kits. To avoid this inconvenience, diapausing pupae, which can be purchased and maintained for a long period in refrigerator without any foods until using for the virus inoculation, are considered as an ideal alternative to larvae. *B. mori* does not diapause at pupal stage but at early embryonic stage. Therefore, diapausing pupae of giant silk moths such as *Hyalophora cecropia* and *Antheraea pernyi*, both of which belong to the genus Saturniidae, were chosen in the pioneered studies (Hellers and Steiner, 1992; Zhang *et al.*, 1992), although these newly developed BEV systems were not practical. In the case of *H. cecropia*, even though high level expression of heterologous genes were obtained by injecting recombinant AcNPVs to the diapausing pupae, it is difficult to get sufficient numbers of diapausing pupae for large scale production. In contrast, *A. pernyi* has a high potential for the large-scale production, because silk production using *A. pernyi* is one of the major industry in the northeastern China. However, *A. pernyi* is not susceptible to AcNPV infection and there was no established cell line supporting replication and plaque purification of *A. pernyi* NPV (AnpeNPV).

Recently an *A. pernyi* cell line, NISES-AnPe-428 (AnPe), was established (Inoue and Hayasaka, 1995). We have demonstrated that AnPe cells support replication and plaque-

purification of the wild type AnpeNPV, indicating that the cell line would be a breakthrough for developing a novel BEV system for *in vivo* production using diapausing *A. pernyi* pupae (Wang *et al.*, 2000) and succeeded to construct a transfer vector plasmid pApCH1, which enable to express foreign genes under the strong polyhedrin promoter of AnpeNPV (Kobayashi *et al.*, 2001).

In this paper, we describe the construction of a recombinant AnpeNPV expressing the *Escherichia coli* β -galactosidase gene (*lacZ*) and compare the efficiency of β -galactosidase production in both infected AnPe cells and diapausing *A. pernyi* pupae with those in other BEV systems using AcNPV, BmNPV and *Hyphantria cunea* NPV (HycuNPV) (Takenaka *et al.*, 1999).

Materials and Methods

Bacteria, insects and insect cell lines

Competent *E. coli* strain XL1-Blue cells (Stratagene) were used for plasmid DNA transformations. Diapausing pupae of *A. pernyi* were obtained from Shenyang Agricultural University in October 1997, stored in the refrigerator at 5°C and used for virus infection in May 1998. Fifth instar larvae of *B. mori* were obtained from Katakura-Kogyo Co. Ltd. Five lepidopteran insect cell lines, NISES-AnPe-428 (AnPe), BmN4, IPLB-Sf9 (Sf9), BTI-TN5B1-4 (High5) and FRI-SpIm-1229 (SpIm), were maintained in TC-100 medium supplemented with 10% fetal bovine serum (FBS) (GIBCO/BRL), Sf-900 II medium (GIBCO/BRL) and/or EXCELL-405 (JRH Biosciences) at 27°C.

Plasmids, transfer vectors and viral DNA

The transfer vectors, pApCH1 (Kobayashi *et al.*, 2001), pBM030 (Maeda, 1989), pAcYM1 (Matsuura *et al.*, 1987) and pHcMU2 (Shinoda *et al.*, 2001), were used for the construction of recombinant NPVs expressing *lacZ* gene under the polyhedrin promoters as described later. Viral DNA genomes of wild type AnpeNPV A (Wang *et al.*, 2000), BmNPV T3 (Maeda *et al.*, 1985) and HycuNPV A (Takenaka *et al.*, 1999) were prepared from purified virions in culture supernatants of infected cells by sucrose

density gradient centrifugation (O'Reilly *et al.*, 1992). As AcNPV DNA genome, BaculoGold linearized DNA (Pharming) was used.

DNA manipulations

All plasmid DNA recombination techniques were essentially as described by Sambrook *et al.* (1989). Restriction enzymes and other DNA modifying enzymes were purchased from Takara-Shuzo Co. Ltd.

Construction of recombinant NPVs expressing *lacZ* gene

The *E. coli* β -galactosidase gene cassette (*lacZ*) (3.1 kbp) contained in the plasmid LacZ/pTZ18 (7.2 kbp) (Kamita *et al.*, 1993) was slightly modified to use in this study. Briefly, the LacZ/pTZ18 was digested with *Hind*III and, after blunting reaction, further digested with *Xba*I. Then the 3.1 kbp *Hind*III (blunted)-*Xba*I fragment containing *lacZ* gene without the translation initiation ATG codon was ligated with the transfer vector pBM030 which was digested with *Eco*RV and *Xba*I. In the resulting recombinant transfer vector pBMLacZ, the ATG sequence in *Nco*I site just upstream of *Eco*RV site become in frame with *lacZ* ORF and function as a translation initiation codon (Fig. 1a). This slight change in the amino acid sequence at the N-terminus of β -galactosidase (Fig. 1b) did not affect the enzymatic activity. From pBMLacZ, modified *lacZ* gene cassette (3.2 kbp) was excised by digesting with *Bgl*II and *Bam*HI and ligated with the transfer vector pAcYM1 digested with *Bam*HI or, after blunting, ligated with pApCH1 digested with *Sma*I and with pHcMU1 digested with *Sma*I. By these ligation reactions, recombinant transfer vectors, pAcLacZ, pApLacZ and pHcLacZ, were obtained (Fig. 1a).

To generate recombinant NPVs expressing *lacZ* gene under the control of the polyhedrin promoter (Ppolh), each combination of recombinant transfer vector and viral DNA (pBMLacZ+BmNPV DNA, pAcLacZ+BaculoGold linearized DNA, pApLacZ+ AnpeNPV DNA and pHcLacZ+ HycuNPV DNA) was cotransfected into the respective host cell line (BmN4, Sf9, AnPe and SpIm) by lipofection method, essentially as

described by Kobayashi and Belloncik (1993). In general, 5 μ g of vector and 1 μ g of viral DNA (0.5 μ g for BaculoGold) were mixed with 8 μ l of Lipofectin Reagent (GIBCO/BRL) in 1 ml of serum-free TC-100 medium and added to 5×10^5 cells seeded in each well of 6-well plate (Sumitomo Bakelite). After incubation at 25°C for 8 hours, the lipofection mixture was replaced with 2 ml of fresh TC-100 medium containing 10% FBS and cultured for one week at 27°C. Then the culture supernatants containing recombinant NPVs generated by the homologous recombination between the vector and viral DNA in the transfected cells were collected and subjected to the plaque assay (Kobayashi and Belloncik, 1993). After 5 days of plaque development, 50 μ g of 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal) was added to agarose overlay per each well of 6-well plate and recombinant NPVs (BmLacZ, AcLacZ, AnpeLacZ and HycuLacZ) were obtained by screening for blue-colored and polyhedra-negative plaques. Each putative recombinant was subjected to at least three rounds of plaque purification to obtain genetic homogeneity. The insertion of *lacZ* at the polyhedrin locus in each recombinant viral DNA was confirmed by Southern blot hybridization. The purified recombinant NPVs were amplified using the respective host cells, and the infected culture supernatants containing virions were harvested and, after measuring virus titers, stored as virus stocks at -80°C until use.

Virus infection and sample preparation

To compare the expression efficiency of *lacZ* gene by AnpeLacZ with those by other recombinant NPVs (BmLacZ, AcLacZ and HycuLacZ) both *in vitro* and *in vivo*, following virus infection experiments were performed.

For *in vitro* expression, AnPe, BmN4, Sf9 and SpIm cells cultured in TC-100 medium with 10% FBS and/or Sf900II medium and High5 cells cultured in EXCELL-405 medium were seeded at 5×10^5 cells with 2 ml of culture medium in each well of 6 well-plates, respectively. Then, the cells in each well were infected with 5×10^5 plaque forming unit (PFU), multiplicity of infection (MOI) = 1, of the corresponding



Fig. 1 Schematic illustrations of recombinant transfer vector plasmids, pBMLacZ, pApLacZ, pAcLacZ and pHcLacZ, used for the construction of recombinant NPVs expressing *lacZ* gene (a) and N-terminal sequence comparison between *E. coli lacZ* gene product and modified *lacZ* gene product (b).

recombinant NPVs, AnpeLacZ to AnPe cells, BmLacZ to BmN4 cells, AcLacZ to Sf9 and High5 cells and HycuLacZ to SpIm cells, respectively. After infection, cells were cultured at 27°C and infected cells in one well of each experimental regime were harvested with the medium every 2 days until 12 days post-infection (until 18 days for AnPe cells cultured in Sf-900II medium). The harvested cells and medium were then separated by low-speed centrifugation (1,700×g for 5 min). The cell pellets were resuspended in 0.5 ml of Lysis buffer (0.25M Tris HCl, pH 8) and disrupted by two cycles of freezing and thawing. The insoluble cell debris was pelleted by microcentrifugation (10,000×g for 10 min) and the supernatant was collected as cell lysates. Both the medium and cell lysates were used for β -galactosidase assay as well as SDS-PAGE and Western blot analyses

For *in vivo* expression, diapausing *A. pernyi* pupae stored at 4°C about 7 months and 5th instar *B. mori* larvae within one day after ecdysis were used for injection of 5×10^5 PFU of ApLacZ and BmLacZ per insect, respectively. Pupae and larvae of mock infection as well as wild type virus infection were also prepared by injecting an equivalent amount (50 μ l) of fresh TC-100 medium per insect and by injecting 5×10^5 PFU of AnpeNPV and BmNPV per insect, respectively. After injection, both pupae and larvae were kept at 25°C. Six pupae (3 females and 3 males) were sampled every 3 days until 18 days post-injection when the infected pupal tissues were completely liquefied, while 3 larvae were sampled every day until 5 days post-injection when the infected larvae were completely dying. After measuring body weights, the pupae and larvae were individually homogenized in 5 ml ice cold phosphate buffered saline (PBS) (pH 6.8) containing 0.5% phenylthiourea. The homogenates were centrifuged at 1700 μ g for 5 min and the supernatant was collected as insect extracts. The extracts were used for β -galactosidase assay as well as SDS-PAGE and Western blot analyses.

β -galactosidase assay

The level of β -galactosidase activity was

determined by using β -Galactosidase Assay Kit (Invitrogen) as follows. First, each sample was mixed with ortho-nitrophenyl- β -D-galactopyranoside (ONPG) and after 30 min incubation at 37°C, absorbance at 420 nm (OD₄₂₀), a peak absorbance of the hydrolyzed ONPG was measured. Then, β -galactosidase activity (units = nmols of ONPG hydrolyzed/min) per 1ml of each sample was calculated by the following formula.

$$\beta\text{-galactosidase activity (units/ml)} = \text{OD}_{420} \times \frac{V (\mu\text{l})}{t (\text{min}) \times v (\mu\text{l}) \times 0.0045}$$

where V is the volume of a reaction mixture (800 μ l), t is the time of reaction at 37°C (30 min), v is the volume of a sample (1-10 μ l), 0.0045 is the extinction coefficient (ml/nmol). Finally the calculated values were converted into activities of β -galactosidase produced per 1 ml cell culture or 1g insect and compared one another.

SDS-PAGE and Western blot analyses

Samples prepared from cell cultures and insects were analyzed by electrophoresis in 8% SDS-polyacrylamide gels (Laemmli, 1970). Gels were stained with Coomassie brilliant blue or used for Western blot analysis

In Western blot analysis, proteins separated in the gel were transferred onto PVDF membrane (BioRad) using Transblot SD Cell (BioRad) according to the manufacturer's instruction. After blocking with 0.1% Gelatin in PBS, the membrane was probed with a 1:1000 dilution of rabbit anti- β -galactosidase serum (Funakoshi). Then the membrane was washed and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Tago) and visualized with ImmunoStain Kit (Konica).

Results

Expression of β -galactosidase gene in AnPe cell culture

When AnPe cells cultured in TC-100 medium supplemented with 10% FBS were infected with AnpeLacZ at MOI of 1, the cells

showing typical cytopathic effects except the absence of polyhedral inclusion body formation appeared and increased from 3 days post-infection (p.i.). At 10 days pi, most of the cells in the culture were disrupted. The similar infection process was also observed for AnPe cells cultured in Sf-900II medium, although the process proceeded more slowly and it took around 14 days until the most of the cells in the culture were disrupted.

The temporal changes of β -galactosidase activity in the AnPe cell culture infected with AnpeLacZ were investigated at an interval of 2 days (Table 1). For the cells cultured in TC-100 (+ 10% FBS) medium, the activity became measurable from 4 days p.i. and the intracellular activity reached a peak at 8 days p.i., while the activity in the medium largely increased from 6 to 10 days p.i. and approached to a plateau after 10 days p.i., resulting in the highest total activity of 40.9 units/ml cell culture at 12 days p.i. For the cells cultured in Sf-900II medium, the activity became measurable from 6 days p.i. and the intracellular activity reached a peak at 10 days p.i., while the activity in the medium largely increased from 8 to 12 days p.i. and approached to a plateau after 14 days p.i., resulting in the highest total activity of 59.9 units/ml cell culture at 18 days p.i.

The molecular mass of the *lacZ* gene

product was calculated as 116,219 Da from the deduced amino acid sequence. SDS-PAGE analysis of the AnpeLacZ-infected AnPe cells and culture supernatants revealed that there is an obvious band with a molecular mass of ca. 116 kDa, which was not detected in the mock-infected and wild-type AnpeNPV-infected cell culture (Fig. 2a). The band specifically reacted with a rabbit anti- β -galactosidase serum (Fig. 2b), demonstrating that the *lacZ* gene inserted in the recombinant AnpeNPV genome was expressed in the infected AnPe cells.

Expression of β -galactosidase gene in A. pernyi pupae

Diapausing *A. pernyi* pupae stored at 5°C for 7 months were injected with AnpeLacZ at 5×10^5 PFU/pupa. The infected pupal body became soft and color of the head end changed from white to black at 9 days p.i. The pupal tissues were completely liquefied at 15 days p.i. These pathogenic symptoms were quite similar to those observed in the wild-type AnpeNPV-infected pupae.

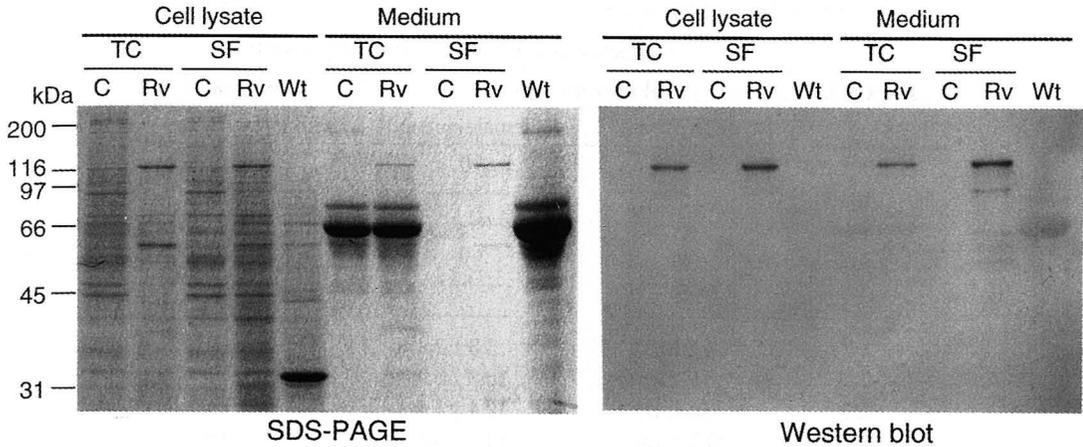
The temporal changes of β -galactosidase activity in the pupae infected with AnpeLacZ were investigated at an interval of 3 days (Table 2). In both male and female pupae, the activity was detected from 3 days p.i., greatly increased until 9 days p.i. and reached a peak

Table 1. Comparison of temporal changes in the activity of β -galactosidase produced by several *in vitro* BEV systems.

Days p. i.	β -galactosidase activity (unit/ml cell culture)																				
	AnPe			BmN4			SpIm			Sf9			High5								
	TC*		SF*	TC		SF	TC		SF	TC		SF	EX*								
	cell*	sup*	total*	cell	sup	total	cell	sup	total	cell	sup	total	cell	sup	total	cell	sup	total	cell	sup	total
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0.2	0.2	0.1	0.2	0.3	1.5	1.6	3.1	1.3	1.0	2.3	
4	0.4	0.2	0.6	0	0	0	0.7	14.4	15.1	1.9	2.6	4.5	12.9	16.4	29.3	5.4	10.7	16.1	60.0	17.9	77.9
6	5.9	2.6	8.5	0.3	0.7	1.0	0.2	12.2	12.4	6.3	21.8	28.1	3.6	40.6	44.2	8.0	35.6	43.6	55.5	150.3	205.8
8	12.1	16.9	29.0	1.1	7.0	8.1	0	12.0	12.0	6.8	42.4	49.2	1.5	53.5	55.0	2.6	47.1	49.7	41.4	284.6	326.0
10	1.3	34.4	35.7	6.1	21.0	27.1	0	10.0	10.0	3.3	65.3	68.6	0.8	71.6	72.4	0.4	66.0	66.4	28.7	206.6	235.3
12	0.4	40.5	40.9	2.6	39.1	41.7	0	9.8	9.8	1.0	52.7	53.7	0.3	60.1	60.4	0.2	47.8	48.0	19.9	199.1	219.0
14	—	—	—*	1.8	49.0	50.8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16	—	—	—	0.5	54.4	54.9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18	—	—	—	0.2	59.7	59.9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

*) TC, TC-100 medium + 10% FBS; SF, Sf900II medium; EX, EX-CELL 405 medium; cell, cell lysate; sup, culture medium; total = cell + sup; —, not done.

a. AnPe cell culture



b. *A. pernyi* pupae

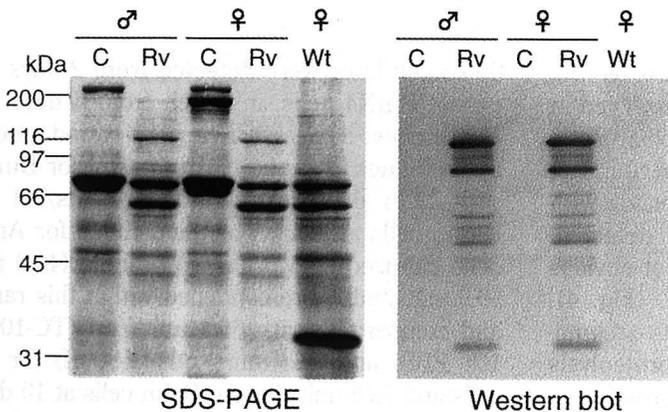


Fig. 2 SDS-PAGE and Western blot analyses of cell lysates and media of AnpeLacZ-infected AnPe cell culture at 12 days p.i. (TC) and 18 days p.i. (SF) (a) and homogenates of *A. pernyi* pupae at 12 days p.i. (b). TC, TC-100 medium (+10% FBS). SF, SF-900II medium. C, mock-infected, Rv, AnpeLacZ-infected, and Wt, wild-type AnpeNPV-infected AnPe cell culture. For wild-type AnpeNPV infection, AnPe cells were cultured in TC-100 medium (+10% FBS). An equivalent quantity of cell lysates and media of AnPe cell culture, and an equivalent quantity of homogenates of *A. pernyi* pupae were used for the analyses.

or a plateau at 15 days p.i. The highest activities of female and male pupae were 14.6×10^2 units/g pupa at 18 days p.i. and 11.7×10^2 units/g pupa at 15 days p.i., respectively. No increase of β -galactosidase activity was detected in both mock-infected and wild type AnpeNPV-infected pupae. Mock-infected pupae normally eclosed to adults between 15 and 18 days after injection.

SDS-PAGE analysis of the AnpeLacZ-infected *A. pernyi* pupae at 12 days p.i. revealed the presence of ca. 116 kDa polypeptide as was detected in the AnpeLacZ-infected AnPe cell culture and again it was not detected in the mock-infected and wild-type AnpeNPV-infected pupae (Fig 2b). In addition, severe degradation of the major pupal proteins such as arylphorin (ca. 75kDa) and female-specific vitellogenin

Table 2. Comparison of temporal changes in the activity of β -galactosidase produced by two *in vivo* BEV systems.

Days p. i.	β -galactosidase activity* ($\times 10^2$ unit/g insect)		
	<i>Antheraea pernyi</i>		<i>Bombyx mori</i>
	male pupae	female pupae	5th instar larvae
0	0	0	0
1	—————*	—————	0
2	—————	—————	0
2	0	0	0.4 \pm 0.1
4	—————	—————	4.6 \pm 0.9
5	—————	—————	10.1 \pm 1.8
6	4.5 \pm 0.6	3.3 \pm 2.3	—————
9	8.0 \pm 3.4	10.8 \pm 0.9	—————
12	9.3 \pm 1.1	12.4 \pm 1.4	—————
15	11.7 \pm 1.5	14.3 \pm 3.3	—————
18	10.8 \pm 1.6	14.6 \pm 1.0	—————

*) β -galactosidase activity, average \pm standard deviation of 3 insects; ———, not done.

and vitellin (ca. 200kDa) (Yokoyama *et al.*, 1993, Kajiura *et al.*, 1998) was also observed in virus-infected pupae. In Western blot analysis using a rabbit anti- β -galactosidase serum, not only the 116 kDa band but several bands with smaller molecular masses were detected, although these smaller bands were not obvious in the infected pupae at 6 days p.i. (Fig. 4), indicating that the *lacZ* gene products accumulated in the pupae suffered serious proteolysis as observed in other major pupal proteins in the late stage of infection.

Comparison of productivities between *AnpeNPV* vector system and other BEV systems

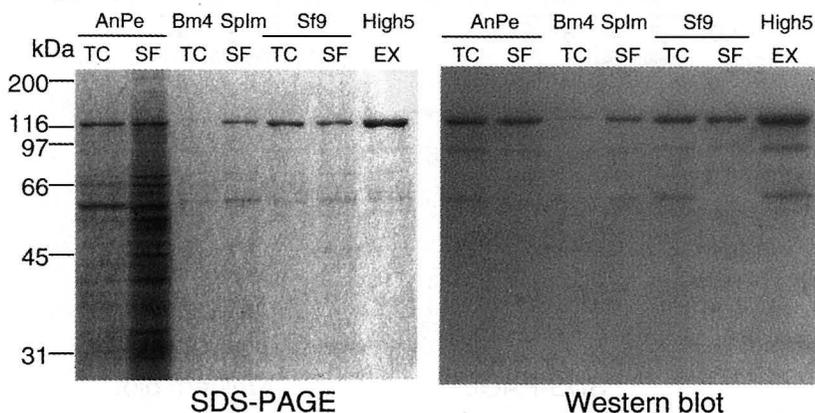
In order to evaluate the efficiency of the foreign protein production in the newly developed *AnpeNPV* vector system, we have also expressed the same *lacZ* gene under the control of the respective polyhedrin promoters in other BEV systems and compared their β -galactosidase activities.

For comparison of the productivity among *in vitro* systems, each cell line was infected with a corresponding recombinant NPV (AcLacZ for Sf9 and High5 cells, BmLacZ for BmN4 cells and HycuLacZ for SpIm cells) at a MOI of 1 and the temporal change of β -galactosidase activity in each cell culture was investigated at an interval of 2 days (Table 1). The activities in

these cell lines were detected from 2 days p.i. except BmN4 cells, in which from 4 days p.i. The highest total activities were varied among the cell lines between 15.1 units/ml for BmN4 cells at 4 days p.i. and 326.0 units/ml for High5 cells at 8 days p.i., and those for AnPe cells cultured in two kinds of media (40.9 and 59.9 units/ml) were contained within this range and comparable with 66.4 units/ml (TC-100 + 10% FBS) and 72.4 units/ml (Sf-900II) for Sf9 cells and 68.6 units/ml for SpIm cells at 10 days p.i. SDS-PAGE and Western blot analyses revealed that the identical 116 kDa polypeptides of β -galactosidase were produced and accumulated in all of the cell cultures infected with recombinant NPVs expressing the same *lacZ* gene (Fig. 3). In addition, several immunoreactive bands with smaller molecular masses were also detected among these cell cultures, especially for High5 cells, indicating proteolytic degradation of the recombinant protein occurred more or less in any of the *in vitro* production systems.

To compare the productivity of *in vivo* production system using *A. pernyi* diapausing pupae, the 5th instar larvae of *B. mori* was injected with BmLacZ at 5×10^5 PFU/larva. In contrast with the slow increase of β -galactosidase activity in *A. pernyi* pupae, the activity increased quickly in *B. mori* larvae from 3

a. Cell lysate



b. Medium

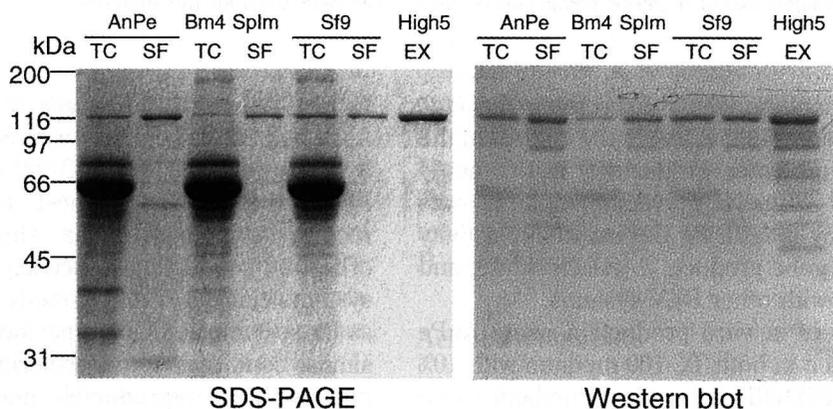


Fig. 3 SDS-PAGE and Western blot analyses of cell lysates (a) and media (b) of several lepidopteran insect cell cultures infected with recombinant NPVs on the day showing the highest β -galactosidase activity. AnPe, AnpeLacZ-infected AnPe cell cultures at 12 days p.i. (TC) and 18 days p.i. (SF), Bm4, BmLacZ-infected BmN4 cell culture at 4 days p.i., SpIm, HycuLacZ-infected SpIm cell culture at 10 days p.i., Sf9, AcLacZ-infected Sf9 cells at 10 days p.i. (TC and SF), and High5, AcLacZ-infected High5 cell culture at 8 days p.i. EX, EX-CELL 405 medium. The other abbreviations are the same as in Fig. 2. An equivalent quantity of cell lysates and media of each cell culture were used for the analyses.

days p.i. and reached the highest value of 10.1×10^2 units/g larva at 5 days p.i., just prior to the death (Table 2). *B. mori* larvae showed the similar highest activity to *A. pernyi* male pupae but less than female pupae. SDS-PAGE and Western blot analyses revealed that the identical 116 kDa polypeptides of β -galactosidase as well as its degradatives with smaller molecular masses accumulated in both *B. mori* larvae

and *A. pernyi* pupae (Fig. 4). However, the degradation at late stage of infection in *B. mori* (5 days p.i.) was not so severe as in *A. pernyi* (15 days p.i.).

Discussion

In this paper, we have first reported that the successful construction and purification of

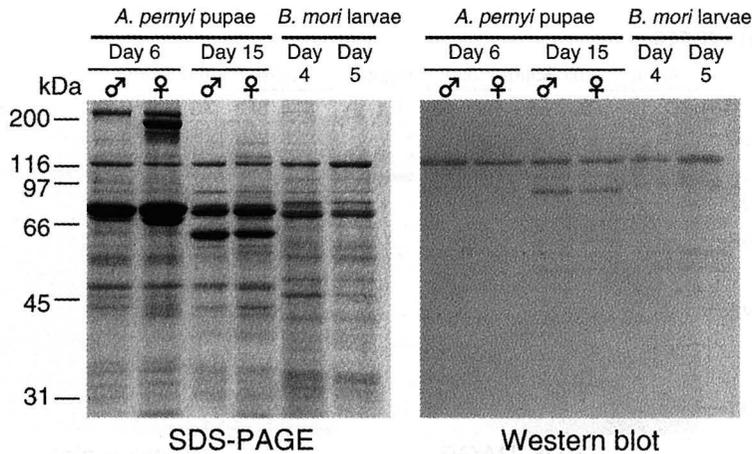


Fig. 4 SDS-PAGE and Western blot analyses of homogenates of AnpeLacZ-infected *A. pernyi* pupae at 6 and 15 days p.i. and BmLacZ-infected *B. mori* larvae at 4 and 5 days p.i.. An equivalent quantity of homogenates of *A. pernyi* pupae and *B. mori* larvae were used for the analyses.

a recombinant AnpeNPV expressing the *lacZ* gene as a foreign gene under the control of the polyhedrin promoter. Productivity in this newly established AnpeNPV vector system was estimated by measuring the enzymatic activity of the *lacZ* gene product, β -galactosidase, and comparing with other BEV systems.

Levels of *in vitro* production using AnPe cells cultured in both TC-100 medium with 10% FBS and Sf-900II serum-free medium were higher than BmN4 cells, comparable with Sf9 and SpIm cells and lower than High5 cells (Table 1), and the speeds of production in the AnPe cell culture, especially with Sf-900II medium, were slower than the other *in vitro* BEV systems. Our results agreed with the previous reports that High5 cells in monolayer culture showed higher efficiency of recombinant protein production than any other AcNPV-susceptible lepidopteran insect cells including Sf9 cells (Davis *et al.*, 1993; Wickham and Nemerow, 1993). Many factors including cell density, virus titer, cell growth rate, medium composition, culture method and virus-cell interaction must be involved in the temporal pattern of the *lacZ* gene expression in each cell culture. Among these factors, virus-cell interaction has been well studied and considered as the major determinant of the productivity.

In fact, although the speeds of production in AnPe and Sf9 cell cultures with Sf-900II medium were slower than with TC-100 medium (+10% FBS), the similar highest activities were recorded with both media. Thus, the relative order of production efficiency among BEV systems elucidated in this study would be valid as far as comparisons are performed under the similar conditions to our experiments, but may not be always reproducible under completely different conditions. Strokovskaya *et al.* (1996) established a BEV system using another *A. pernyi* cell line (MCAp-1) and *Malacosoma neustria* NPV (MnNPV). They reported lower level of β -galactosidase activity in MnNPV-MCAp-1 cell system than in AcNPV-Sf9 cell system. It is not certain whether MCAp-1 cells are less productive than AnPe cells. Infection of AnpeLacZ instead of recombinant MnNPV might improve the β -galactosidase production in MCAp-1 cells as in AnpeNPV-infected AnPe cells.

In vivo production using *A. pernyi* pupae, β -galactosidase activity per gram insect in female pupae was about 1.3-fold higher than that in male pupae on average (Table 2). The fact that the female pupa (about 12g) is 1.5-fold heavier than the male pupa (about 8g) indicates that one female pupa is quantitatively equivalent

to two male pupae in the protein production. As described for polyhedral inclusion body (PIB) production in gypsy moth larva by Shapiro (1986), more virus and more recombinant protein are produced in a greater biomass attained by the female. *B. mori* 5th instar larvae showed similar productivity to *A. pernyi* male pupae and less than female pupae, suggesting that the potential advantages of female pupae for recombinant protein production. As observed in AnPe cells, the β -galactosidase activity in *A. pernyi* pupae increased significantly slower than in *B. mori* larvae. The slow production of recombinant protein seemed to be an identical characteristic of AnpeNPV vector system, probably reflecting moderate speeds of AnpeNPV multiplication in *A. pernyi* cells. In addition, the virus multiplication may be further delayed under physiological conditions in diapausing pupae, such as slow diffusion of free virions in pupal body fluids and retarded viral DNA replication in diapausing cells.

An obvious drawback of the slow production in diapausing pupae was significant degradation of recombinant protein at later stage of infection (Fig. 2). However, similar proteolytic degradation of recombinant protein was commonly, although less than in *A. pernyi* pupae, observed not only in *B. mori* larvae (Fig. 4) but also more or less in all the insect cell cultures (Fig. 3). It is well known that cysteine protease (cathepsin) gene (*v-cath*) is encoded in the baculovirus genome and expressed at late stage of infection (Ohkawa *et al.*, 1994, Hawtin *et al.*, 1997). Thus, it is likely that the degradation of β -galactosidase had become most remarkable by longer incubation time with cathepsin in *A. pernyi* pupae. In the BmNPV vector system, proteolytic degradation of recombinant protein was virtually suppressed by deleting the *v-cath* coding region from the viral DNA genome (Suzuki *et al.*, 1997). We have already identified a *v-cath* homolog in AnpeNPV genome (data not shown) and started to construct the similar protease-free AnpeNPV.

Although it is not easy to compare productivities between *in vitro* and *in vivo* systems (Maeda, 1989), our results offer a rough estimate by comparing the highest activity of

β -galactosidase in 1 ml of each cell culture and in 1 g of each insect (Table 1, 2). According to the estimate, *in vivo* systems using *A. pernyi* pupae and *B. mori* larvae are much more productive than any of *in vitro* systems. Not only better protein yields at less cost, but also more efficient post-translational modification may be achieved by differentiated insect cells *in vivo* (Maeda, 1989, Hellers and Steiner, 1992). We will further investigate the properties of post-translational modifications in *A. pernyi* pupae. The advantages of *in vivo* production, however, may be counterbalanced by the disadvantages, such as laborious task to tend living insects and difficulty in downstream purification (O'Reilly *et al.*, 1992). The former disadvantage is completely no problem for *A. pernyi* pupae, because we just purchase diapausing pupae and store them in the refrigerator for a long-time (over one year) until use for the recombinant protein production. Altogether, our results suggested that the AnpeNPV vector system using *A. pernyi* diapausing pupae has a potential to become more practical protein production system than *B. mori* larvae.

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