Antheraea pernyi Nuleopolyhedrovirus Mutants Lacking Functional Cathepsin and/or Chitinase Genes Suppress Proteolysis and Liquefaction of Virus-infected Diapausing *A. pernyi* Pupae

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Antheraea pernyi Nuleopolyhedrovirus Mutants Lacking Functional Cathepsin and/or Chitinase Genes Suppress Proteolysis and Liquefaction of Virus-infected Diapausing *A. pernyi* Pupae

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Abstract **In order to improve the yield of recombinant proteins produced in a baculovirus expression vector (BEV) system using diapausing pupae of the Chinese oak silkworm, *Antheraea pernyi*, and *A. pernyi* nucleopolyhedrovirus (AnpeNPV) by avoiding proteolytic degradation and liquefaction of virus-infected pupae mainly caused by collaborative action of two virus-encoded enzymes, cathepsin and chitinase, we have engineered AnpeNPV DNA genome so as to lack the functional cathepsin gene (*v-cath*) and/or chitinase (*chiA*) gene. By replacing the *v-cath* gene region of AnpeLacZ, a recombinant AnpeNPV expressing the *Escherichia coli* β-galactosidase gene (*lacZ*) under the control of the polyhedrin promoter, with a marker gene cassette composed of the *Drosophila* 70 kDa heatshock protein (Dhsp70) promoter and the *Aequorea victoria* green fluorescent protein (GFP) cDNA, we have obtained a *v-cath* lacking mutant AnpeLacZ (*v-cath*$^-$). We have also obtained another mutant AnpeLacZ (*v-cath*$^-$/*chiA*$^-$) by replacing the region containing both the *v-cath* and *chiA* genes of AnpeLacZ with the same marker gene cassette. At later stages of infection, both protease activity and degradation of β-galactosidase detected in AnpeLacZ-infected cell culture and pupae were virtually suppressed in the mutants-infected counterparts. In addition, no obvious liquefaction occurred in mutants-infected pupae. However, β-galactosidase activities of AnPe cells infected with AnpeLacZ and two mutant viruses reduced at similar speeds, indicating the enzyme inactivation at later stage of infection is caused by factors other than the cathepsin activity. The results suggested that the disruption of *v-cath* is sufficient to avoid both proteolysis and liquefaction but is not enough to enhance the stability of expressed foreign proteins at later stages of infection.**
Key words: baculovirus, *Antheraea pernyi*, NPV, cathepsin, chitinase

Introduction

A newly established baculovirus expression vector (BEV) system using the diapausing pupae of Chinese oak silkworm, *Antheraea pernyi*, and *A. pernyi* nucleopolyhedrovirus (AnpeNPV) showed higher protein production capability than the previously established BEV system using the larvae of mulberry silkworm, *Bombyx*

mori, and *B. mori* NPV (BmNPV) (Kobayashi *et al.*, 2001; Huang *et al.*, 2001). An additional advantage of the AnpeNPV system over the BmNPV system is utilization of diapausing pupae, which can be stored for a long time (over one year) in the refrigerator until use for the protein production by recombinant virus injection. The time course of protein production in the diapausing *A. pernyi* pupae is quite different from that in *B. mori* larvae. In the diapausing pupae, the polyhedrin promoter-mediated protein production proceeded slowly and reached maximal levels around 15 days post-infection (p.i.) when the pupae were obviously liquefied, while the protein production in the larvae reached maximal level at 5 days post-infection just before the death and liquefaction. During the slow production in diapausing pupae, proteolytic degradation proceeded concurrently. As a result, at later stages of infection, a significant part of recombinant proteins accumulated in the pupae were remarkably degraded (Huang *et al.*, 2001).

The proteolytic degradation of foreign gene products and liquefaction of host insect tissues during virus pathogenesis are common phenomena observed in the BEV systems and mainly caused by collaborative action of cysteine protease (cathepsin) and chitinase encoded in the baculovirus genome as revealed by following studies. Ohkawa *et al.* (1994) reported that BmNPV encodes a cysteine protease belonging to the papain superfamily. Slack *et al.* (1995) also reported that *Autographa californica* NPV (AcNPV) encodes a papain type cysteine protease (cathepsin) with cathepsin L-like characteristics. The cathepsin digests the proteinaceous components of the insect cadaver during liquefaction, including the internal organs as well as the protective sheath of protein surrounding the chitinous elements of the cuticle (Smith *et al.*, 1981). Larvae infected with virus lacking a functional cathepsin gene (*v-cath*) do not undergo melanization and remain fully intact following death (Slack *et al.*, 1995). The cathepsin simultaneously also digests foreign proteins produced in insect culture cells and larvae infected with a recombinant baculovirus expressing foreign gene. Using BmNPV lacking the *v-cath* gene, recombinant proteins were efficiently produced and the degradation was suppressed in *B. mori* larvae (Suzuki *et al.*, 1997). In addition, Hawtin *et al.* (1995) identified a chitinase gene (*chiA*) in AcNPV genome. The chitinase is a late viral gene product targeted to the endoplasmic reticulum (ER) of infected cells (Thomas *et al.*, 1998) and is speculated to have a primary role to degrade cuticular chitin of the host insects at the end of the infection process. Hawtin *et al.* (1997) have demonstrated that liquefaction of AcNPV-infected insects is dependent on the integrity of virus-encoded *chiA* and *v-cath*. Recently, AcNPV chitinase is found to be required for the processing of the viral cathepsin (Hom and Volkman, 2000).

Thus, for improving the yield of recombinant proteins by avoiding the protein degradation and tissue degeneration in the diapausing *A. pernyi* pupae infected with recombinant AnpeNPV, genetic engineering of virus genome to disrupt *v-cath* gene or both *v-cath* and *chiA* genes is considered as the most fundamental and effective strategy. We have already cloned and sequenced both *v-cath* and *chiA* genes identified in the physical map of AnpeNPV genome (Huang *et al.*, 2002). In this study, we have generated two mutant AnpeNPVs lacking the functional *v-cath* gene and both the *v-cath* and *chiA* genes, respectively. Using these mutants, effects on proteolytic degradation and liquefaction of *A. pernyi* pupae were examined.

Materials and Methods

Bacteria, plasmids, insects, cells, viruses and restriction enzymes

Competent cells of *E. coli* strains TOP10F' (Invitrogen) and JM110 (Stratagene) were used for plasmid DNA transformation. Plasmids pBluescript II (Stratagene) and pCR2.1 (Invitrogen) were used for DNA cloning. A *gfp* gene cassette contained in the palsmid pDhspGFP (Kobayashi, unpublished) that is constructed to express the *Aequorea victoria* green fluorescence protein (GFP) cDNA under the control of the 70-kDa heat shock protein promoter derived from *Drosophila melanogaster* was used as a marker gene. Diapausing pupae of *A. pernyi* were provided by Dr. Zenta Kajiura

(Shinshu University) and stored for 8 months at 5℃. The NISES-AnPe-428 (AnPe) cells isolated from *A. pernyi* (Inoue and Hayasaka, 1995) were maintained in TC-100 medium supplemented with 10% fetal bovine serum (FBS) (Sigma) or SF-900II (Invitrogen) at 27℃ and used for DNA transfection, virus infection and plaque assay. Virus DNA was isolated from the recombinant AnpeNPV, AnpeLacZ, which express the *E. coli* β-galactosidase gene (*lacZ*) under the control of the polyhedrin promoter (Huang *et al.*, 2001) and used to construct two mutants of AnpeLacZ, AnpeLacZ (*v-cath*$^-$) and AnpeLacZ (*v-cath*$^-$/*chiA*$^-$). All of restriction enzymes (Takara Bio) were used according to the manufacturer's instruction.

Construction of two mutant AnpeNPVs lacking the functional v-cath *gene and both* v-cath *and* chiA *genes*

As previously reported (Huang *et al.*, 2002), both the *v-cath* and *chiA* genes are located in a head-to-head arrangement within a *Hind*III fragment (3874bp) (accession number AB072731) contained in the AnpeNPV *Pst*I A fragment (9.1 kbp). To disrupt the *v-cath* gene and both *v-cath* and *chiA* genes in the DNA genome of AnpeLacZ, two transfer vector plasmids, pApv-cath$^-$ and pApv-cath$^-$/chiA$^-$, were constructed (data not shown).

To generate two mutants, AnpeLacZ (*v-cath*$^-$) and AnpeLacZ (*v-cath*$^-$/*chiA*$^-$), the DNA genome of AnpeLacZ was cotransfected with each of the transfer vectors, pApv-cath$^-$ and pApv-cath$^-$/chiA$^-$, into AnPe cells by lipofection method (Kobayashi and Belloncik, 1993). Mutant viruses expressing the *gfp* gene, generated by homologous recombination between virus DNA and transfer vectors, were isolated and purified by plaque assay methods. Plaques formed by mutants were identified by fluorescent microscopic observation of green fluorescence emitted by GFP produced in the mutant-infected cells. LacZ gene expression was confirmed by blue color developed after adding 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal).

As illustrated in Fig. 1, in the AnpeLacZ (*v-cath*$^-$) genome, the *chiA* gene was intact and the *v-cath* gene was unfunctional because of the replacement of the promoter and N-terminal coding regions of *v-cath* gene with the *gfp* gene cassette, while in the AnpeLacZ (*v-cath*$^-$/*chiA*$^-$) genome, both the *v-cath* and chiA genes were

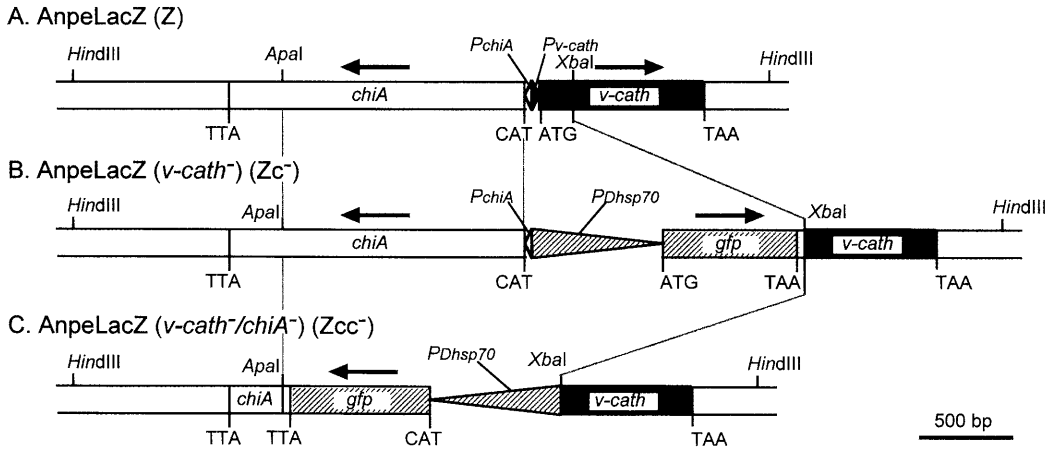

Fig. 1. Schematic representation of the cathepsin gene (*v-cath*) and chitinase gene (*chiA*) region in genome DNA of the three recombinant AnpeNPV. (A): AnpeLacZ (Z). (B): AnpeLacZ (*v-cath*$^-$) (Zc$^-$). (C): AnpeLacZ (*v-cath*$^-$/*chiA*$^-$) (Zcc$^-$). *Pv-cath*, *PchiA* and *PDhsp70* represent the promoter sequences for *v-cath*, *chiA* and 70-kDa heat shock protein gene of *Drosophila*. The positions of translation initiation codons (ATG, or CAT for complementary) and stop codons (TAA, or TTA for complementary) for *v-cath*, *chiA* and *gfp* genes are indicated. Sites for several restriction enzymes are also indicated. Arrows indicate the directions of transcription of each gene. A scale bar represents 500 bp. For details, see Materials and Methods.

unfunctional because the promoter and N-terminal coding regions of both genes were replaced with the *gfp* gene cassette. By PCR amplification using viral DNA genome as a template, the replacement of target gene(s) by the *gfp* gene cassette in both mutants were confirmed (data not shown).

Virus infection, sample preparation and measurement of β-galactosidase activity

Virus infection, sample preparation and the measurement of β-galactosidase activity were as described in Huang *et al.* (2001). In brief, AnPe cells seeded in the 6-well plate (5×10^5 cells) or T-75 flask (3×10^6 cells) were infected with each virus at plaque forming unit (PFU) of 5×10^5 (multiplicity of infection, MOI = 1). For analyses, cells and media of virus-infected cultures were collected separately. The *A. pernyi* pupae were infected by injection of each virus with 5×10^5 PFU per insect. Each infected *A. pernyi* pupa was homogenized in 5ml ice-cold 10mM sodium phosphate buffer (PBS) (pH 6.8) containing 0.5% phenylthiourea after measurement of body weight and the supernatant collected after centrifugation at 1700 × g for 5 min was used as a sample for analyses.

SDS-PAGE and Western blot analyses

SDS-PAGE and Western blot analyses were performed as described previously (Huang *et al.* 2001). Each sample was adjusted to contain 0.2 units of β-galactosidase and a cysteine protease inhibitor, trans-epoxysuccinylamido (4-guanidino)-butane (E-64), was added with a final concentration of 30μg/ml after sample preparation.

Measurement of cathepsin activity

Activities of cathepsin in virus-infected *A. pernyi* pupae and AnPe cells were measured using azocoll (Sigma) as a substrate by a modified method described by Kobayashi *et al.* (1985). 150μl of the supernatant of pupal homogenates that was equivalent to ca. 0.1g of pupal body weight or 150μl of the supernatant of AnPe cell lysates that were prepared at 5 days post-infection, just prior to cell lysis caused by virus multiplication, by sonicating cell suspension in 0.3 ml PBS (pH 6.8) was added to 500μl of azocoll (6mg/ml in 100mM succinic acid-NaOH, pH 4,5), and incubated at 37℃ for 3 h. The reaction was stopped by adding 750μl of 10% SDS, mixed and centrifuged (13,000×g, 5min). The absorbance at 520 nm of each supernatant was measured. All samples were assayed in the presence and absence of E-64.

Results and Discussion

Characterization of two mutants of AnpeLacZ lacking cathepsin gene and both cathepsin and chitinase genes

Proteolytic activities in AnPe cells (TC-100 medium with 10% FBS) at 5 days post-infection (p.i.) and *A. pernyi* pupae at 15 days p.i. were analyzed (Fig. 2). In AnpeLacZ-infected AnPe cells and *A. pernyi* pupae, higher levels of protease activity which were strongly suppressed by E-64, a cysteine protease inhibitor, were detected, while in those infected with two mutants, levels of protease activity were quite low and addition of E-64 did not decrease the levels, indicating the most parts of protease activity found in the AnpeLacZ-infected cells and pupae can be attributable to the virus cathepsin.

Liquefaction of AnpeLacZ-infected *A. pernyi* pupae started from 9 days p.i., when the white color at the top of head turned black, reflecting the color changes of the internal body fluid caused by the tissue degeneration. Then the integument of body was gradually getting fragile and, finally, became easily torn when handled at 15 days p.i. In contrast, the pupae infected with AnpeLacZ (*v-cath*$^-$) and AnpeLacZ (*v-cath*$^-$/*chiA*$^-$) were intact and no obvious symptoms of liquefaction were observed at 15 days p.i., and even up to 25 days p.i (data not shown). There was no significant difference in the appearance of infected pupae between two mutants.

β-galactosidase production by two mutant viruses

SDS-PAGE and Western blot analyses of culture supernatants of AnPe cells and homogenates of *A. pernyi* pupae revealed that the proteolytic degradation of β-galactosidase observed in the samples infected with AnpeLacZ was significantly suppressed in those infected

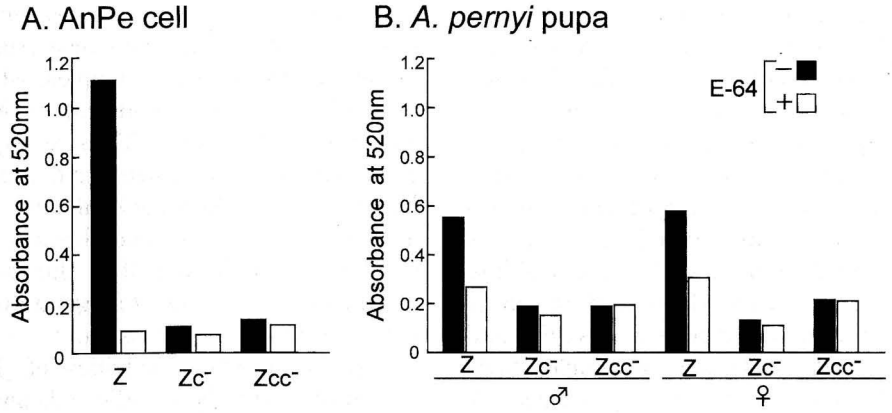

Fig. 2. Protease activity in virus-infected AnPe cells and *A. pernyi* pupae assayed by azocoll as a substrate. (A): AnPe cell lysates at 5 days p.i. (B): *A. pernyi* pupa homogenates at 15 days p.i. Closed bars and open bars indicate absorbance at 520 nm assayed without and with the cysteine protease inhibitor (E-64), respectively. Z: AnpeLacZ, Zc⁻: AnpeLacZ (*v-cath*⁻), Zcc⁻: AnpeLacZ (*v-cath*⁻/*chiA*⁻).

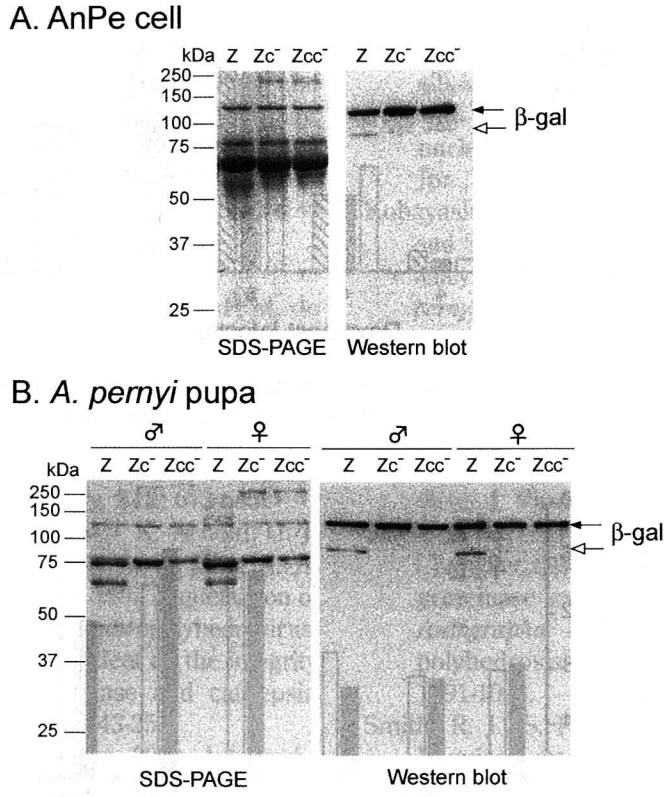

Fig. 3. SDS-PAGE and Western blot analyses of virus-infected AnPe cells and *A. pernyi* pupae. (A): AnPe cell lysates at 10 days p.i. (B): *A. pernyi* pupa homogenates at 15 days p.i. Black and white arrows indicate intact β-galactosidase (116 kDa) and its major degraded product (90 kDa), respectively. In all samples, 30μg/ml of cysteine protease inhibitor (E-64) was added. Z: AnpeLacZ, Zc⁻: AnpeLacZ (*v-cath*⁻), Zcc⁻: AnpeLacZ (*v-cath*⁻/*chiA*⁻).

with the mutants (Fig. 3). In fact, in addition to the 116-kDa of intact β-galactosidase polypeptide, a 90-kDa polypeptide was also detected abundantly in the AnpeLacZ infected AnPe cells and pupae by Western blot analysis using anti-β-galctosidase antibody. The 90-kDa polypeptide and other minor immunoreactive bands observed in the AnpeLacZ-infected samples were considered as degraded β-galactosidase polypeptides, and were decreased or disappeared in the both AnpeLacZ (*v-cath*$^-$)- and AnpeLacZ (*v-cath*$^-$/*chiA*$^-$)-infected samples. SDS-PAGE analysis suggested not only β-galactosidase but also other protein components were subjected to the degradation in the AnpeLacZ-infected samples and were remaining intact in the mutant viruses-infected samples, because lower molecular polypeptides observed in the AnpeLacZ-infected samples disappeared in the mutant viruses-infected samples, while higher molecular polypeptides absent in the formers presented in the latters. Thus, as expected, we could suppress the proteolytic degradation and liquefaction of AnpeLacZ-infected pupae by disrupting the virus-encoded *v-cath* gene. Our results also indicated that the *v-cath* gene disruption without the *chiA* gene disruption is sufficient for the suppression.

However, when activities of β-galactosidase produced in both AnPe cells and *A. pernyi* pupae infected with AnpeLacZ, AnpeLacZ (*v-cath*$^-$) and AnpeLacZ (*v-cath*$^-$/*chiA*$^-$) were analyzed and compared (Fig. 4, 5), levels and temporal changes of β-galactosidase activity were similar

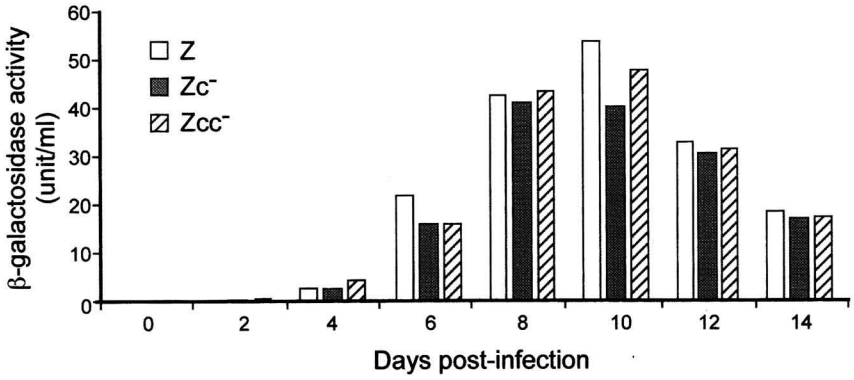

Fig. 4. Temporal changes of β-galactosidase activities in virus-infected AnPe cell cultures. Z: AnpeLacZ, Zc$^-$: AnpeLacZ (*v-cath*$^-$), Zcc$^-$: AnpeLacZ (*v-cath*$^-$/*chiA*$^-$).

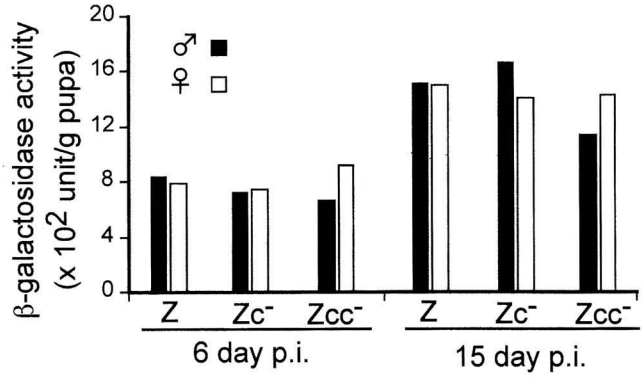

Fig. 5. Comparison of β-galactosidase activities in virus-infected *A. pernyi* pupae (averages of two individuals) at 6 and 15 days p.i. Z: AnpeLacZ, Zc$^-$: AnpeLacZ (*v-cath*$^-$), Zcc$^-$: AnpeLacZ (*v-cath*$^-$/*chiA*$^-$).

and, in AnPe cells, decline of activity subsequently to the peak level at 10 days p.i. was not decelerated by the infection of mutant viruses, in spite of the significant reduction of β-galactosidase degradation. One possible explanation for the apparent discrepancy between activity and structure of β-galactosidase is that the decline of enzymatic activity is mainly caused by factors other than the virus-encoded cathepsin. Further investigations are required to confirm the possibility. Except this, all of the results indicated that AnpeNPV mutant lacking the functional *v-cath* gene is useful to produce large amounts of undegraded recombinant proteins, especially those hypersensitive to the cathepsin, in the diapausing *A. pernyi* pupae.

Acknowledgments

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