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# Influences of chitinase gene deletion from BmNPV on the cell lysis and host liquefaction

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**Summary.** A *Bombyx mori* nucleopolyhedrovirus (BmNPV) mutant with chitinase gene (*chiA*) deletion (Bm*chiA*$^-$) was constructed. The effects of *chiA* deletion on virus infected BmN cells and silkworm larvae were examined. The *egfp* gene expression under the control of the *chiA* promoter, in Bm*chiA*$^-$-infected BmN cells could be detected as early as 8 h p.i. The fluorescence reached its peak at 48 h p.i., and was prevalent until the cell lysis. Cytopathological comparison of the cells infected with wild type and mutant viruses showed that deletion of *chiA* could evidently delay the cell lysis. SDS-PAGE analysis suggested that the content of polyhedrin in Bm*chiA*$^-$-infected cells at 7 days p.i. was about 28% of the total cellular proteins, about 7% lower than that in wild-type BmNPV virus-infected cells. The symptoms of *B. mori* larvae infected with Bm*chiA*$^-$ were different from those of wild-type BmNPV-infected larvae, e.g., clear haemolymph and less degradation of the body after larval death.

## Introduction

Members of the *Baculoviridae* are divided into two genera, *Granulovirus* and *Nucleopolyhedrovirus.* The genus *Nucleopolyhedrovirus* can be further divided into two morphological groups, based on the number of nucleocapsids per virion. The single-nucleopolyhedroviruses (SNPV) contain a single nucleocapsid per virion, whereas the multiple-nucleopolyhedroviruses (MNPV) contain multiple nucleocapsids per virion. Both the SNPVs and MNPVs can contain numerous virions per inclusion body. *Autographa californica* MNPV (AcMNPV) is a representative for MNPVs, and *Bombyx mori* NPV (BmNPV) for SNPVs [6]. Baculoviruses have a circular, double stranded DNA genome. The genomes of these viruses range in size from 80–180 kbp and contain 120–160 ORFs. Lots of information have

been generated through studies on AcMNPV. BmNPV is very closely related to AcMNPV and shares a significant level of homology at the genomic level.

Among the 16 fully sequenced lepidopteran NPV genomes, all but *Adoxophyes honmai* nucleopolyhedrovirus contained an ORF encoding chitinase. Baculovirus *chiA* gene was first identified in AcMNPV genome from AcMNPV-infected *Spodoptera frugiperda* cells [1], the *chiA* is expressed as a late protein (58 kDa) with endo- and exo-chitinase activities [3]. Chitinase [4] and cathepsin (V-CATH), a cysteine protease [9, 12] function together to promote liquefaction of the host after death. The *chiA* gene of AcMNPV can be deleted without affecting virus replication in cultured cells or insects, but the deletion of either *chiA* or *v-cath* abrogates the liquefying process [4, 12]. Chitinase retained high levels of activity between pH 3.0 and 10.0 or very alkaline conditions, as compared with the optimum pH of 4.0∼5.0 for the majority of microbial chitinases [4]. Further analysis demonstrated that AcMNPV chitinase was located within the endoplasmic reticulum (ER) of virus-infected cells, and there was a KDEL ER retention motif at the carboxyl end of the protein [14]. The mutation of the KDEL motif can result in chitinase passing through the secretory pathway and redistribution of chitinase within the cell during virus infection. The biological activity of the KDEL mutant virus in *Trichoplusia ni* was enhanced [10, 11]. But so far, there are no reports on function of *chiA* promoter and effect of *chiA* deletion on the lysis of the cell membrane.

BmNPV is a natural pathogen of the mulberry silkworm, *Bombyx mori*. Subsequently, the BmNPV based expression vector system has gained prominence for over-expression of cloned foreign genes in insect cells and silkworm larvae. The role of virus-encoded cathepsin has been observed in the liquefaction process in BmNPV-infected insect larvae [7].

The *chiA* gene was also found in BmNPV genome [2] and shares high identity with that of AcMNPV. We constructed a *chiA* knock-out mutant virus during this study, in order to understand the functions of *chiA* gene and virus pathogenicity, and thus to improve the expression system for foreign genes in the BmNPV-silkworms. The biological effects of *chiA* gene deletion from the BmNPV genome were also investigated.

## Materials and methods

### *Virus and insect cells*

The *Bombyx mori* cell line, BmN, was maintained at 27 °C in TC-100 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA) as described by Summers and Smith [13] and used for propagation of wild type (wt) BmNPV (Zhenjiang strain) and recombinant BmNPV [8].

### *Construction of chiA deletion mutant*

The *chiA* is located between 97,049 and 98,707 bp in the BmNPV genome (GenBank accession No. L33180) [2], with a transcriptional orientation opposite to the polyhedrin gene. The transfer vector pE*chiA*$^{-}$ was produced by inserting *egfp* and *chiA* flanking sequences (Fig. 1A)

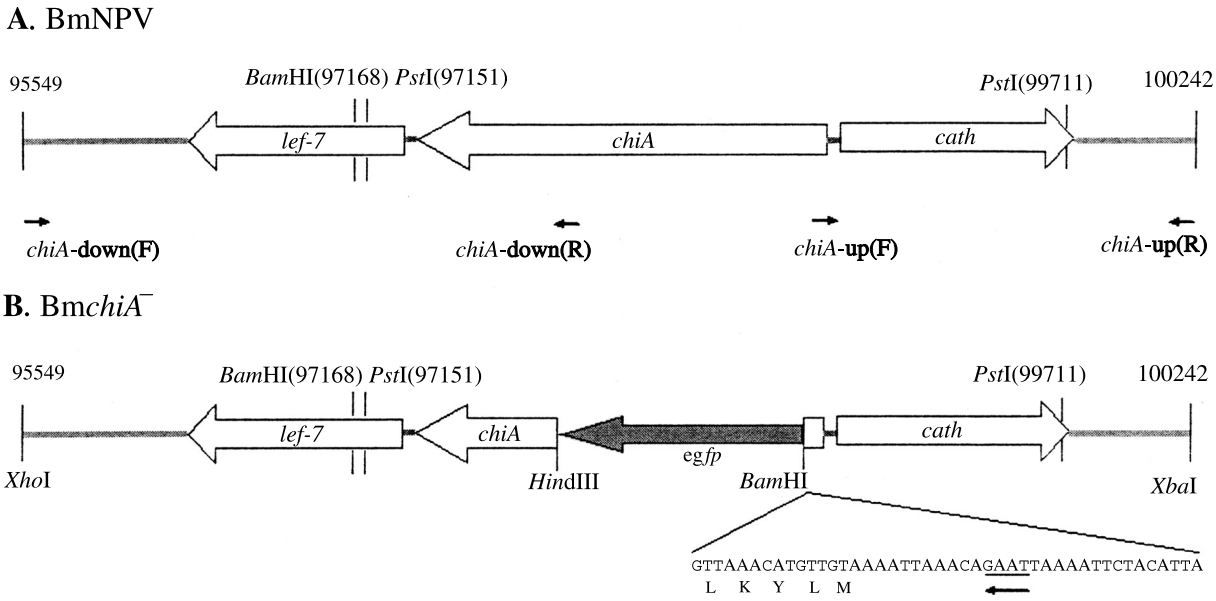


**Fig. 1.** Genomic organizations for *chiA* region of BmNPV and the recombinant virus. **A**, Region of BmNPV genome showing locations and orientation of *lef-7*, *chiA* and *cath* genes. The positions of the PCR primers used to amplify each gene are indicated; **B**, Replacement of portion of *chiA* with *egfp* under *chiA* promoter control to derive Bm*chiA*$^-$ recombinant virus

into the multicloning site (MCS) of pBlueScript SK(+). Firstly, the coding sequence of *egfp* was isolated from pEGFP-N1 [16] by digestion with *Bam*HI and *Hin*dIII and inserted into pBlueScript SK(+), which was digested with *Bam*HI and *Hin*dIII beforehand, to produce pEGFP. Then, a 1550 bp upstream fragment of *chiA* was amplified by PCR using a reverse primer, *chiA*-up(R), 5′-ATCTAGATGCTACAACAACAGC-3′ (corresponding to 100,228–100,242 nt in the BmNPV genome) incorporating a *Xba*I site (underlined), and a forward primer, *chiA*-up(F), 5′-AGGATCCCAATTTGTACAACAT-3′ (corresponding to 98,693–98,707 nt in the BmNPV genome) with a *Bam*HI site (underlined). The amplified fragment was inserted into pGEM-T easy vector (Promega, USA). The insert was retrieved by digestion with *Xba*I and *Bam*HI, and ligated into pEGFP, to produce pEGFP/up. Finally, a 1997 bp downstream fragment of *chiA* was produced by using PCR with two additional oligonucleotides, the forward primer, *chiA*-down (F), 5′-ACTCGAGCGCGCTGCAATAAAC-3′ (95,549–95,563 nt in BmNPV genome) with a *Xho*I site (underlined), and the reverse primer, *chiA*-down(R), 5′-AGAAGCTTGGTCAAACACGGA-3′ (97,533–97,545 nt, in BmNPV genome) with a *Hin*dIII site (underlined). The PCR product was also cloned and the insert was retrieved by digestion with *Xho*I and *Hin*dIII, and then was inserted into pEGFP/up to form the final transfer vector pE*chiA*$^-$ (Fig. 1B).

In the constructed transfer vector, a total of 1147 bp in the central portion of *chiA* (corresponding to the 97,546–98,692 bp in the genome) was replaced by *egfp*, only the first 15 bp and the last 497 bp of *chiA* were retained. The *egfp* was in frame with the first 15 bp and under the control of the *chiA* promoter. The *chiA* flanking sequences along with the retained partial chitinase coding regions were used for the homologous recombination. The transfer vector pE*chiA*$^-$ DNA was co-transfected with wt BmNPV genomic DNA into BmN cells mediated by Lipofectin (Gibco, USA). The recombinant BmNPV, Bm*chiA*$^-$, was isolated by plaque assay under a fluorescent microscope. PCR analysis was performed to identify the genomic DNA derived from viral particles of Bm*chiA*$^-$-infected BmN cells. The primers

used for PCR identification of *egfp* and *chiA* are as follows: *egfp* forward primer, *egfp*(F), 5′-AGGGATCCATGGTGAGCAAGGGCG-3′ (*Bam*HI site underlined), *egfp* reverse primer, *egfp*(R) 5′-CTTAAGCTTACTTGTACAGCTCGTCC-3′ (*Hin*dIII site underlined), *chiA* forward primer, *chiA*(F), 5′-AGGATCCATGTTGTACAAATTG-3′ (*Bam*HI site underlined), *chiA* reverse primer, *chiA*(R), 5′-AGAATTCACAGTTCATCTC-3′ (*Eco*RI site underlined).

Progeny virus was titrated by end point dilution assay. High-titer stocks of recombinant viruses were prepared in BmN cells as described by Summers and Smith [13].

*Time course of egfp expression under the control of chiA gene promoter*

BmN cells were inoculated with recombinant virus Bm*chiA*$^-$ (10 PFU/cell) and photographed under a fluorescent microscope at 0, 2, 4, 6, 8, 10, 12, 14, 16, 24, 30, 36, 48, 72, 96 h post infection (p.i.), respectively.

*Effects of chiA gene deletion on cell lysis and release of polyhedra*

BmN cells were inoculated with BmNPV and Bm*chiA*$^-$ (10 PFU/cell). The polyhedra in virus-infected cells and released into the culture medium were calculated and photographed at 4 days and 7 days p.i., respectively.

*Infection of baculoviruses on silkworm larvae*

Silkworm larvae were injected subcutaneously with 5 μl of BmNPV and Bm*chiA*$^-$ virus inoculum on the first day of the fifth-instar. Infected larvae were reared at 27 °C. The haemolymph was collected at 5 days p.i. The symptoms of infected larvae were observed during infection and after mortality.

## Results

### *Production of BmchiA*$^-$

The transfer vector pE*chiA*$^-$ constructed during this study for homologous recombination was co-transfected with wt BmNPV genomic DNA (Fig. 1A) and introduced into BmN cells. After homologous recombination, the recombinant BmNPV was designated as Bm*chiA*$^-$ (Fig. 1B), and was isolated by identifying the fluorescent plaque. The single fluorescent plaque (Fig. 2A) was obtained after 4 rounds of plaque purification. PCR analysis of the recombinant virus (Bm*chiA*$^-$) genomic DNA further confirmed that the recombinant virus was pure and the recombinant orientation was correct (Fig. 2B). These results also showed that the *chiA* gene is an unessential gene for virus replication in BmN cells. The titers of Bm*chiA*$^-$ and wt-BmNPV had no significant difference, and both were about $10^8$ PFU/ml at 4 days p.i.

### *egfp gene expression under the control of chiA gene promoter*

In AcMNPV, *chiA* is a late gene [3]. In BmN cells infected with BmNPV mutant, *egfp* gene was expressed driven by the promoter of BmNPV*chiA*. To further identify the *chiA* promoter intensity and the initiation time of foreign gene expression, the time course of *egfp* expression was observed under fluorescent microcope at 0, 2, 4, 6, 8, 10, 12, 14, 16, 24, 30, 36, 48, 72, 96 h p.i., respectively. Fluorescence could be observed as early as 8 h p.i. Photographs of *egfp* expression at 0, 8, 12, 24, 36, 48, 72 and 96 h p.i. were shown in Fig. 3. Therefore, it could be concluded

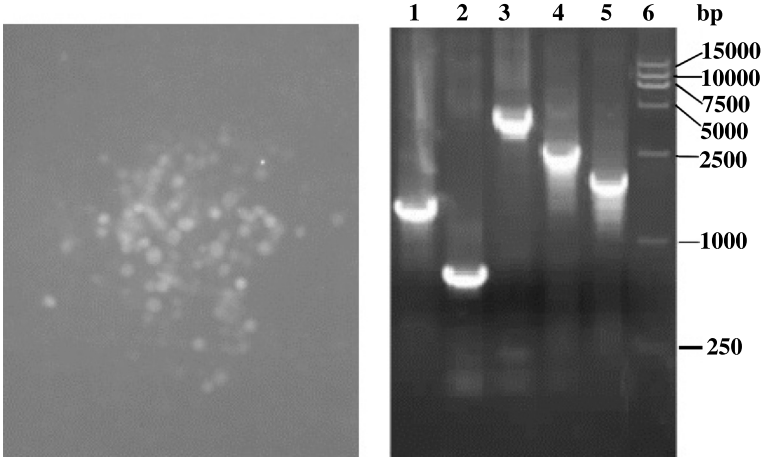


**Fig. 2.** The purification of Bm*chiA*$^{-}$ recombinant virus. **A**, The single fluorescent plaque; **B**, PCR identification of the genomic DNA derived from viral particles of Bm*chiA*$^{-}$-infected BmN cells. *1*, PCR products with *chiA*(F) and *chiA*(R); *2*, PCR products with primers *egfp*(F) and *egfp*(R); *3*, PCR products with primer *chiA*-up(F) and *chiA*-down(R); *4*, PCR products with primers *egfp*(F) and *chiA*-down(R); *5*, PCR products with primers *chiA*-down(F) and *chiA*-down(R); *6*, DNA marker

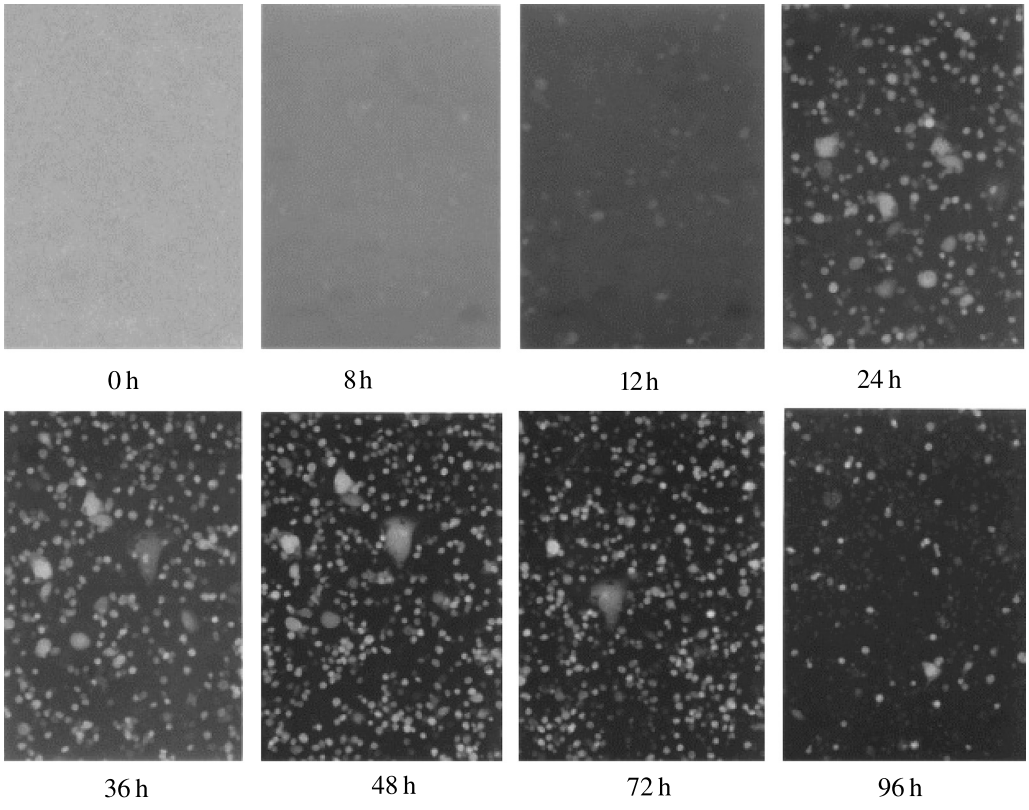


**Fig. 3.** *egfp* expression phase controlled by the promoter of *chiA* in Bm*chiA*$^{-}$-infected BmN cells at 0, 8, 12, 24, 36, 48, 72, 96 h p.i., respectively

that the promoter of *chiA* began to drive *egfp* expression between 6 and 8 h p.i. The fluorescence reached a peak at 48 h p.i., and prevailed at this level until cell lysis at 72 h p.i. These results indicated that BmNPV *chiA* promoter is also a late promoter and can efficiently drive foreign gene expression.

*The effects of BmNPV chiA deletion on cell lysis*

Polyhedra formation in wt BmNPV and Bm*chiA*$^{-}$-infected BmN cells could be observed as early as 48 h p.i. To determine the effects of *chiA* deletion on virus infected BmN cells, the release of polyhedra from lysed cells into the medium were examined. About 5% of polyhedra were released from lysed cells infected with wt BmNPV into the culture medium at 4 days p.i. (Fig. 4A), and fifteen percent of polyhedra were released into the medium at 7 days p.i. (Fig. 4C). While the Bm*chiA*$^{-}$-infected cells were almost intact at 4 days p.i., only about 0.3% of polyhedra were released into the culture medium even 7 days p.i. (Fig. 4B and D). The cytopathological observations of the cells infected with wild type and mutant viruses showed that chitinase could influence cell lysis. The expression levels of

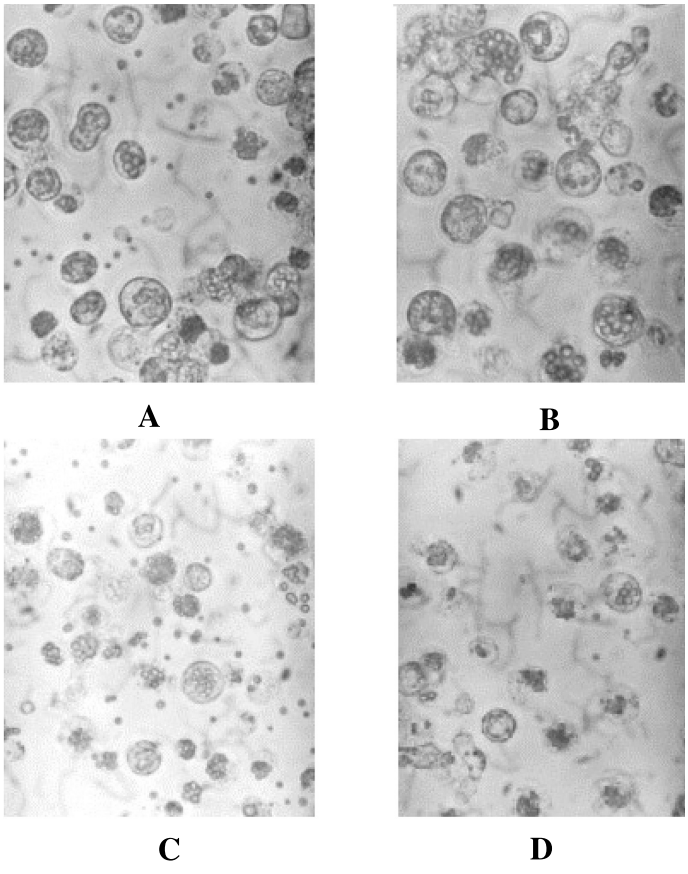


**Fig. 4.** BmN cells infected with wt BmNPV and Bm*chiA*$^{-}$. **A**, wt BmNPV-infected BmN cells 4 days p.i.; **B**, Bm*chiA*$^{-}$-infected BmN cells 4 days p.i.; **C**, wt BmNPV-infected BmN cells 7 days p.i.; **D**, Bm*chiA*$^{-}$-infected BmN cells 7 days p.i.

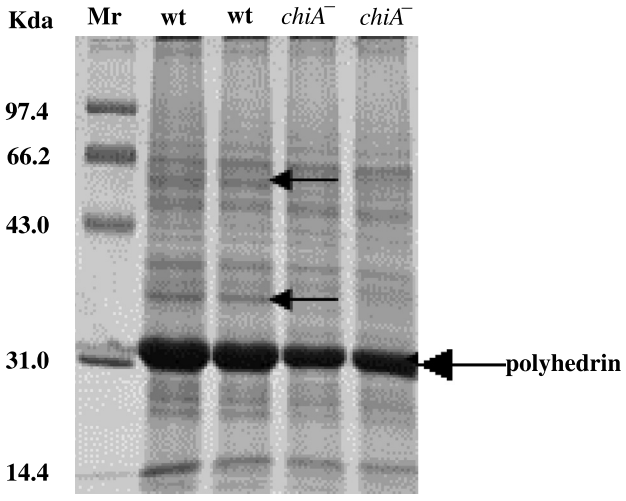


**Fig. 5.** SDS-PAGE analysis of wt-BmNPV and Bm*chiA*$^{-}$-infected Bm cells at 7 days p.i. in 12% gels. *Mr*, Marker proteins; *wt*, the total protein from cells infected with wt-BmNPV; *chiA*$^{-}$, the total protein from cells infected with Bm*chiA*$^{-}$. 25 μg of protein was applied for each lane. The arrows represent the possible bands of chitinase (59.8 kD) and a *chiA* related protein (36 kD)

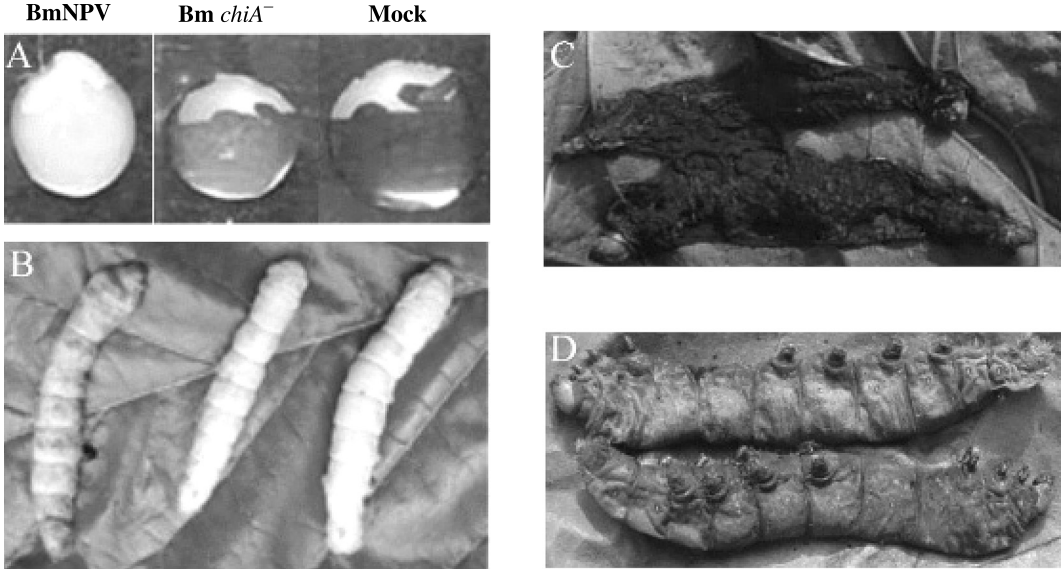


**Fig. 6.** Comparison of the haemolymph and liquefaction time of the fifth instar larvae. **A**, the haemolymph of the fifth instar silkworm larvae infected with wt BmNPV and Bm*chiA*$^{-}$, or mock infected (mock) at 5 days p.i.; **B**, Individual larva infected with wt BmNPV and Bm*chiA*$^{-}$, or mock infected immediately after death; **C**, wt BmNPV-infected 5th instar larvae 11 days p.i. (6 days after death), the larval skin liquefied rapidly; **D**, Bm*chiA*$^{-}$-infected 5th instar larvae 11 days p.i. (6 days after death), the larval skin remained firm

polyhedrin protein in the wild type and mutant virus infected-cells were further investigated by SDS-PAGE analysis. SDS-PAGE profile of virus-infected cells at 7 days p.i. showed that the production of polyhedrin protein in wt BmNPV-infected

cells was higher than in Bm*chiA*$^-$-infected cells (Fig. 5). Thin layer scanning of SDS-PAGE pattern indicated that polyhedrin protein accounted for about 28% of the total protein in Bm*chiA*$^-$-infected cells, and 35% in the BmNPV-infected cells. These results suggest that *chiA* gene deletion may influence the production of polyhedra in Bm*chiA*$^-$-infected cells.

### *Effects of BmchiA$^-$ on liquefaction of larvae*

All the larvae injected with wt BmNPV and Bm*chiA*$^-$ died between 120 and 144 h p.i. However, there were various differences between wt BmNPV and Bm*chiA*$^-$ infected larvae. The haemolymph of silkworm larva infected with wt BmNPV was obviously more turbid than that infected with Bm*chiA*$^-$ at 5 days p.i. (Fig. 6A). The larvae infected with wt BmNPV turned pale and creamy in color prior to death, then blackened and liquefied rapidly after death (Fig. 6B). The larvae infected with Bm*chiA*$^-$ almost retained a normal white skin color and did not melanize or melt after death and the larval skin remained firm even after 6 days after death (Fig. 6C and D).

## Discussion

During this study we constructed a BmNPV recombinant (Bm*chiA*$^-$), in which part of the *chiA* coding region was replaced by *egfp* gene. The BmNPV *chiA* promoter in its natural locus was chosen to drive *egfp* expression. EGFP began to express between 6 h and 8 h p.i. and reached a peak at 48 h p.i. Therefore, BmNPV *chiA* gene is a late gene as that in AcMNPV [3] and is unessential for virus replication. The promoter of *chiA* gene can be used to drive foreign gene expression.

BmN cells infected with Bm*chiA*$^-$ almost did not lyse and release polyhedra even at late stages, in contrast to cells infected with BmNPV (Fig. 4). Polyhedrin production in Bm*chiA*$^-$-infected cells was lower than that in BmNPV-infected cells (Fig. 5). SDS-PAGE gel revealed two protein bands, with sizes of about 59 and 36 kDa, respectively, which could not be seen in Bm*chiA*$^-$ -infected cells (Fig. 5) as compared with wt BmNPV-infected cells (Fig. 5, indicated with arrows). The larger band of 59 kDa was thought to be the chitinase, whose putative size was 59.8 kDa. The smaller band was assumed to be a protein which is directly or indirectly related to chitinase, such as precursor of V-CATH (proV-CATH, with a size of 36.9 kDa), or a protein closely related to cathepsin (V-CATH). Chitinase has been shown to be essential for correct folding of proV-CATH in the endoplasmic reticulum in AcMNPV-infected cells, thus it may serve as a precursor for proV-CATH here [5].

Previous studies indicated that chitinase enters the secretory pathway of virus-infected cells following cleavage of a eukaryotic signal peptide [15] and then retained in the endoplasmic reticulum (ER) until terminal cell lysis results in its release [15]. Therefore, it can be assumed that chitinase might have no direct role in the release of polyhedra from virus-infected cells. On the other hand, V-CATH could not be processed properly in the absence of chiA [5]. Ohkawa et al. [7] and Slack et al. [12] proposed that in tissue culture V-CATH might contribute to

lysis of infected cells, which typically occurs about 72 h p.i. Thus, the absence of cell lysis in *chiA* deficient mutant may pass through a pathway in which V-CATH seems to be involved.

In our study, we observed that the larvae infected with Bm*chiA*$^{-}$ failed to liquefy after death when compared with wt BmNPV-infected silkworm larvae. This indicates that the chitinase of BmNPV has the same function like that in AcMNPV [4]. The haemolymph of Bm*chiA*$^{-}$-infected larvae was found to be more transparent than that infected with wt BmNPV. Similar phenomena was reported in larvae infected with the cysteine protease gene deletion mutant virus [14]. The decrease of the haemolymph turbidity of the larvae infected with *chiA* deletion mutant may be caused by the reduction of cysteine protease, which requires chitinase for the processing of mature V-CATH [5]. Detailed characterization of the pathway in BmN cells and silkworm larvae caused by BmNPV *chiA* deletion still needs to be investigated further.

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