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ORGINAL PAPER

The Effect of a Small Conotoxin-Like *ctx* Gene from *Autographa californica* Nuclear Polyhedrosis Virus (AcMNPV) on Insect Hemolymph Melanization

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Abstract

The conotoxin-like (*ctx*) gene encodes a small cysteine-rich polypeptide in various baculoviruses. Previous research has demonstrated that the product of the *ctx* gene could be purified from insect cells infected by *Autographa californica* nuclear polyhedrosis virus (AcMNPV), but its function was unknown. In this paper, we compared the conserved cysteine motif structure ($CX_3GX_2CX_5CCX_3CX_6C$) of the *ctx* gene in baculoviruses and generated recombinant *Bombyx mori* nuclear polyhedrosis virus (BmNPV) with the BmNPV bacmid system. The recombinant BmNPV contained the *ctx* gene from AcMNPV or a fusion gene of *ctx* with eGFP, respectively. Fluorescence in CTX-eGFP-positive cells was mainly observed on the cell membrane. To gain insight into CTX function, two methods were used to elucidate the affect CTX had on hemolymph melanization *in vivo* and *in vitro* in insect larvae and pupae. The results indicated that CTX abrogates hemolymph melanization; however, the mechanisms require further evaluation.

Key words: AcMNPV, baculovirus, BmNPV, conotoxin, hemolymph, melanization

Introduction

Baculoviruses are major insect pathogens that are characterized by large double-stranded DNA genomes, ranging from 80 to over 180-kb in size. *Bombyx mori* nuclear polyhedrosis virus (BmNPV) and *Autographa californica* NPV (AcMNPV) are two examples of baculoviruses. Both have approximately 128-kb genomes, and both encode approximately 150 genes (Rohrmann, 2011). These genes act during various stages of the baculovirus infection cycle and can be grouped into five functional categories: RNA transcription, DNA replication, structural proteins, auxiliary proteins, and proteins of unknown function (Herniou *et al*., 2003). The genes that are highly homologous to known genes from other species have been well studied; however, the functions of genes with small open reading frames (ORFs) are difficult to identify. In some cases of baculovirus genome analyses, ORFs shorter than 60 aa were neglected. The AcMNPV *ctx* gene was identified as a small cysteine-rich polypeptide (53 aa). Its size and sequence are similar to omega-conotoxins, a class of calcium ion channel inhibitors found in the venom of cone snails (Eldridge *et al*., 1992). Transcriptional assays and product purification of the *ctx* gene by high-pressure liquid chromatography fractionation demonstrated that *ctx* was expressed during the baculovirus infection, but the biological role for *ctx* in AcMNPV infection remains unknown. Interestingly, the BmNPV genome has 90% homology to AcMNPV; however, the *ctx* gene is missing from BmNPV (Olivera *et al*., 1990; Olivera *et al*., 1991).

Because invertebrates lack an adaptive immune response, the innate immune system is crucial to combat microbial infection (Ferrandon *et al*., 2007; Lemaitre and Hoffmann, 2007; Michel and Kafatos, 2005). The synthesis of antimicrobial peptides (AMPs) and activation of the prophenoloxidase system are important components of the humoral response in invertebrate blood cells (Cerenius and Soderhall, 2004; Imler and Bulet, 2005; Williams, 2007). Phenoloxidase (PO) is responsible for enzymatic browning during the growth of the insects and is a key enzyme in the melanization response. Melanization is effective against pathogens, particularly parasites and parasitoid eggs (Barillas-Mury, 2007; Gillespie *et al*., 1997; Kanost *et al*., 2004).

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PO inhibitors are of great interest in the development of new, ecofriendly pesticides (Cerenius *et al*., 2008).

We constructed recombinant BmNPVs that express the AcMNPV *ctx* gene or a fusion gene of *ctx* and eGFP. Decreased melanization was observed when the *B. mori* larvae and pupae were injected with virus expressing *ctx*. These results highlight a novel role for the AcMNPV *ctx* gene in the process of host defense.

Experimental

Materials and Methods

The bacterium *Escherichia coli* DH10Bac/BmNPV (Bm-DH10) was supplied by Prof. E.Y. Park and Prof. K. Maenaka. Plasmids (pFastBac1, pFastBac1-*ie1*, and pBacPAK-eGFP) were constructed in our laboratory. FuGENe6 transfection reagent was purchased from Roche Applied Science, USA. Grace's insect cell culture medium (GIBCO) was purchased from Invitrogen. *B. mori* larvae and pupae were supplied by Dr. Bing Li (Soochow University, China).

The *B. mori* cell line, BmN originating from the ovary, was preserved in our laboratory and cultured at 27°C with TC-100 medium (AptliChem) with 10% FBS (GIBCO).

Preparation of recombinant baculoviral vectors. With AcMNPV genomic DNA as a template, *ctx* was amplified with the following primers: Forward, 5'-AGG GGA TCC ATG CAA ATC AAA ACT GTA C-3'; Reverse, 5'-TGG GGT ACC TTG TGG TAA GCA ATA ATT AAA TAT G-3'. *Bam*HI and *Kpn*I sites are underlined.

The PCR conditions were one cycle at 94°C for 4 min, 30 cycles at 94°C for 20 s, 62°C for 30 s, and 72°C for 30 s, and 1 cycle at 72°C for 7 min. The *ctx* PCR products were digested with restriction enzymes (*Bam*HI and *Kpn*I) and ligated into pBacPAK-eGFP, which was cut with the same enzymes, to generate pBacPAK-*ctx*-eGFP. The *ctx*-eGFP was excised from pBacPAK-*ctx*-eGFP by digestion with *Bam*HI and *Eco*RI, and was subsequently cloned into the *Bam*HI-*Eco*RI sites of pFastBac1 to generate pFB1-*ph-ctx*-eGFP. Additionally, we cloned *ctx* into the *Bam*HI-*Kpn*I sites of pFastBac1 and pFastBac1-*ie1* to generate pFB1-*ph-ctx* and pFB1-*ie1-ctx*, respectively.

Isolation and verification of recombinant bacmid. The plasmids pFB1-*ph-ctx*-eGFP, pFB1-*ph-ctx* and pFB1-*ie1-ctx* were transformed into *E. coli* Bm-DH10, where transposition occurred. After 4-h incubation at 37°C in SOC medium, transformed cells were plated onto media containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml Bluo-gal, and 40 μg/ml isopropyl-*β*-D-thiogalactopyranoside (IPTG). The plates were incubated at 37°C for a minimum of 24 h. White colonies resistant to kanamycin, gentamicin, and tetracycline were selected. Isolated colonies were streaked onto fresh plates to verify the phenotype. PCR was used to confirm the presence of the construct, we used standard M13 primers. The PCR conditions were one cycle at 94°C for 5 min, 30 cycles at 94°C for 45 s, 58°C for 45 s, and 72°C for 3 min 30 s, and 1 cycle at 72°C for 10 min.

Baculovirus transduction. BmN cells were seeded onto 24-well plates at a density of 1×10^5 per well and incubated for 24 h at 27°C. Recombinant bacmids, confirmed by PCR, were transfected into BmN cells using the transfection reagent to generate the recombinant viruses BmpFB1-*ph-ctx*-eGFP, BmpFB1-*ie1-ctx* and BmpFB1-*ph-ctx*, respectively.

Determination of hemolymph melanization. To characterize CTX activity, 4 groups of hemolymph were examined by ultraviolet spectroscopy at 460 nm. For each group, insects were injected with 1 out of 5 viral variants: BmpFB1-*ie1-ctx*, BmpFB1-*ph-ctx*, a negative control (wild type BmNPV), a positive control (the medium was saturated with melanization inhibitor phenylthiocarbamide), or a blank control (hemolymph without treatment). Test groups contained *B. mori* larvae (fifth-instar) and pupae along with *Eri* silkworm pupae. There were 30 individuals within each group. Infected larvae were placed on a plastic plate with feed and water and maintained at 27°C. Three to four days later, hemolymph was collected. Hemolymph (100 μl) from each treatment group was collected in a tube and diluted with 900 μl ddH_2O. The absorbance of the hemolymph was measured using a microplate reader using 200 μl solution in a 96-well plate. Experiments were performed in triplicate.

Sequence alignment and phylogenetic analyses. Sequences homologous to AcMNPV *ctx* from NCBI were aligned using the MEGA4 (Tamura *et al*., 2007) built-in ClustalW program (version 4.0) with the default settings. Each sequence was manually examined for its amino acid residues at the conserved sites. The aligned consensus motifs were shaded with the GeneDoc Multiple Sequence Alignment Editor and Shading Utility (Version 2.6.02) (Nicholas *et al*., 1997).

Isolated polypeptide sequences were used to build neighbor-joining (NJ) distance tree using PAUP 4.0 Beta 10 (Swofford, 1998). This tree was based on a step matrix constructed from the Dayhoff PAM 250 distance matrix that was developed by R.K. Kuzoff.

Results

Analysis of the CTX polypeptide. Using protein homologue search, we found 28 CTX polypeptides encoded by 21 baculoviruses; the length of reading

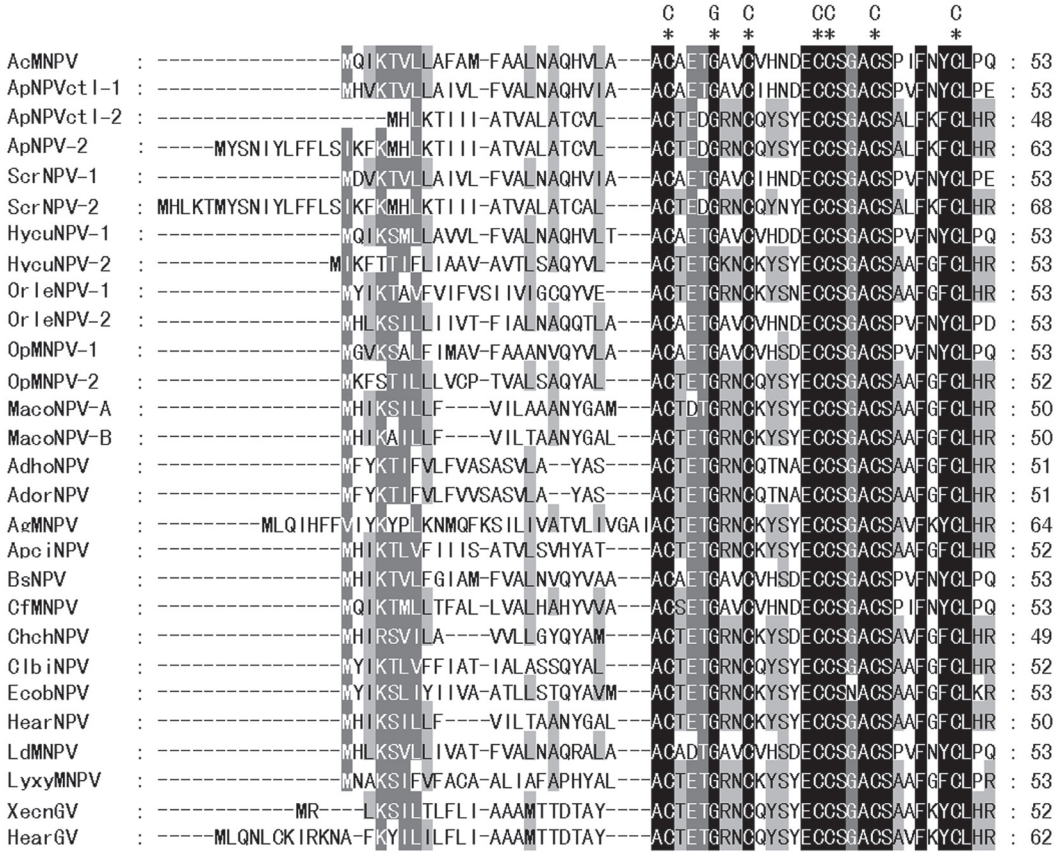

Fig. 1. Alignment of CTX polypeptides from baculoviruses

The consensus sequence is $CX_3GX_2CX_5CCX_3CX_6C$, where X represents any amino acid. Designation of the consensus sequence in shaded areas: black, conserved residues in all species; gray, partially conserved.

AcMNPV (*Autographa californica* nucleopolyhedrovirus, ACNO: NP_054032.1); ApNPV*ctl*-1 (*Antheraea pernyi* NPV, ACNO: YP_611105.1); ApNPV*ctl*-2 (ACNO: YP_001419677.1); ApNPV*ctx*-2 (ACNO: ABQ12349.1); ScrNPV-1 (*Samia cynthia* NPV, ACNO: ADC79662.1); ScrNPV-2 (ACNO: ADC79661.1); HycuNPV-1 (*Hyphantria cunea* NPV, ACNO: YP_473331.1); HycuNPV-2 (ACNO: YP_473311.1); OrleNPV-1 (*Orgyia leucostigma* NPV, ACNO: YP_001650937.1); OrleNPV-2 (ACNO: YP_001651016.1); OpMNPV-1 (*Orgyia pseudotsugata* MNPV, ACNO: NP_046292.1); OpMNPV-2 (ACNO: NP_046186.1); MacoNPV-A (*Mamestra configurata* NPV-A, ACNO: NP_613190.1); MacoNPV-B (*Mamestra configurata* NPV-B, ACNO: NP_689280.1); AdhoNPV (*Adoxophyes honmai* NPV, ACNO: NP_818733.1); AdorNPV (*Adoxophyes orana* NPV, ACNO: YP_002300601.1); AgMNPV (*Anticarsia gemmatalis* NPV, ACNO: YP_803424.1); ApciNPV (*Apocheima cinerarium* NPV, ACNO: ADB84397.1); BsNPV (*Buzura suppressaria* NPV, ACNO: AAC34372.1); CfMNPV (*Choristoneura fumiferana* NPV, ACNO: NP_848442.1); ChchNPV (*Chrysodeixis chalcites* NPV, ACNO: YP_249678.1); ClbiNPV (*Clanis bilineata* NPV, ACNO: YP_717591.1); EcobNPV (*Ecotropis obliqua* NPV, ACNO: YP_874240.1); HearMNPV (*Helicoverpa armigera* multiple NPV, ACNO: YP_002332635.1); LdMNPV (*Lymantria dispar* MNPV, ACNO: NP_047786.1); LyxyMNPV (*Lymantria xylina* NPV, ACNO: YP_003517803.1); XecnGV (*Xestia c-nigrum* granulovirus, ACNO: NP_059275.1); HearGV (*Helicoverpa armigera* GV, ACNO: YP_001649112.1).

frame varied from 48 to 68 aa. There CTX classified as CTL-1, CTL-2 and conotoxin-like 2 were detected in ApNPV (*Antheraea pernyi* NPV). Two CTX are found in the other five kinds of nucleopolyhedrovirus, ScrNPV (*Samia cynthia* NPV), HycuNPV (*Hyphantria cunea* NPV), OrleNPV (*Orgyia leucostigma* NPV), OpMNPV (*Orgyia pseudotsugata* NPV) and MacoNPV (*Mamestra configurata* NPV). The other fourteen baculoviruses encode one CTX polypeptide, which contains two versions of CTX from granulovirus. Based on the alignment of CTX polypeptides from AcMNPV and other baculoviruses, we noticed the most striking similarities in the amount and distribution of cysteine residues (Fig. 1). Although the CTX polypeptides are different in sequence, they share a consensus motif ($CX_3GX_2CX_5CCX_3CX_6C$), including six cysteine residues involved in disulfide bond formation and one glycine between the two N-terminal cysteine residues at precisely the same position; X represents any amino acid. The amino acid residues from 28 CTX polypeptides were used for phylogenetic analysis; the Neighbour-Joining tree is presented on Figure 2.

AcMNPV *ctx* is located between the ORF of *ptp-bro* and ORF4, while ApNPV*ctl*-1 is located between the ORF of *ptp*-1 and *ptp*-2 (Fig. 3). Another *ctx*-like gene, ApNPV*ctx*-2, is located between the ORF of *sod* and *gfg* (Fig. 3). We also amplified two *ctx*-like genes in *Samia cynthia* NPV (ScrNPV) that were almost identical to ctx-like genes from ApNPV (GenBank accession numbers: YP_611105.1 and ABQ12349.1). Although BmNPV and AcMNPV share high sequence homology, the *ctx* gene was not present in BmNPV.

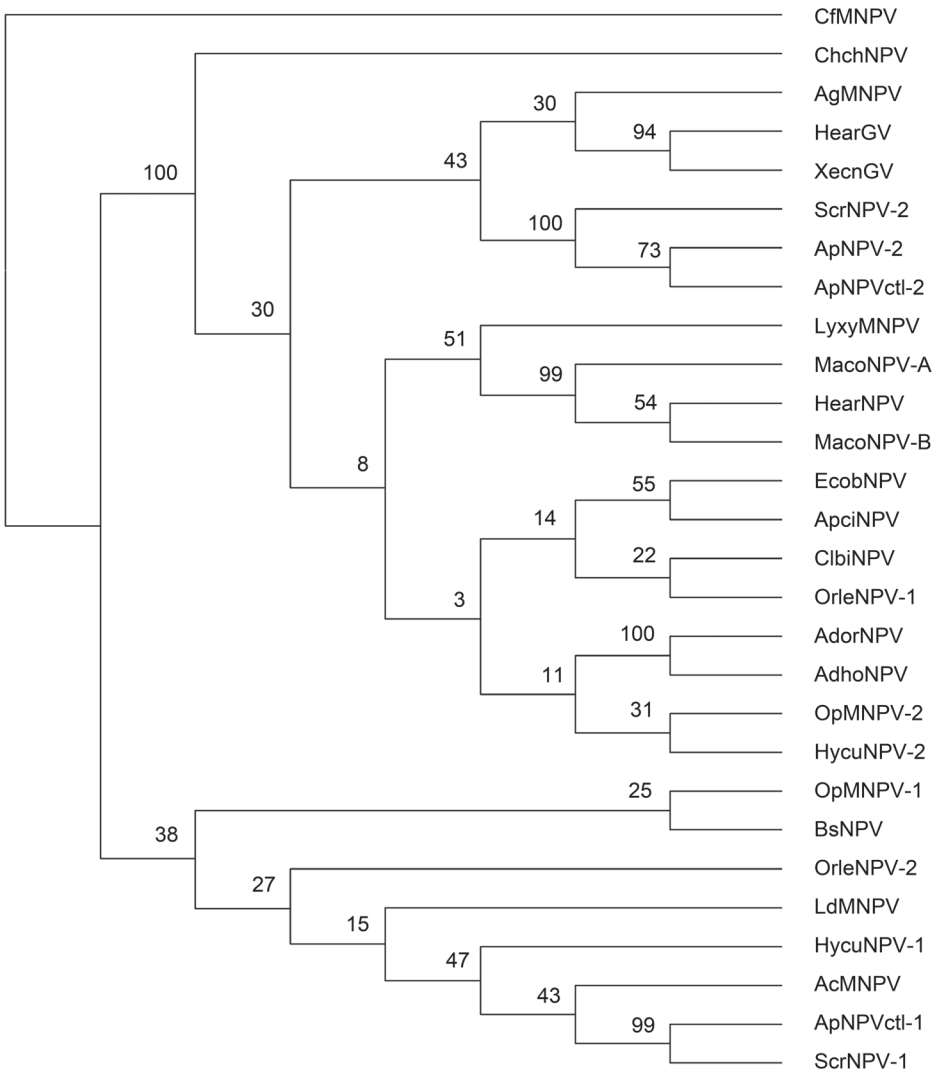

Fig. 2. Phylogenetic characterization of 28 conotoxin-like polypeptides from baculoviruses.
A neighbor-joining (NJ) tree is shown. The CTX from AcMNPV did not form a monophyletic clade with the other 27 sequences and was subject to separate phylogenetic analyses with other members. The numeric values above the nodes indicate bootstrap values.

Identification of the recombinant bacmid. The *ctx* gene was PCR amplified from AcMNPV genomic DNA. The recombinant bacmid DNA is greater than 128 kb in size. Restriction analysis is difficult to perform with DNA of this size. To verify the presence of the foreign fragment in the recombinant bacmid, PCR was used. The bacmid contains M13 priming sites flanking the mini-attTn7 site, which target this site for bacterial transposition and facilitates PCR analysis. Accordingly, the PCR product of the parent bacmid was 300 bp, whereas the PCR product of the recombinant bacmid was 2300 bp plus the size of the insert fragment. Therefore, the PCR products of the recombinant bacmid (transposed with pFB1-*ph-ctx*-eGFP, pFB1-*ph-ctx* and pFB1-*ie1-ctx*) were approximately 3.2, 2.4 and 3.1 kb, respectively, which verified the construction of the recombinant bacmids.

Subcellular localization of CTX-GFP fusion protein. To track the CTX protein, BmN cells were transfected with the recombinant bacmid pFB1-*ph-ctx*-eGFP. Green fluorescence appeared in infected BmN cells 72 h after infection. Cells were subsequently stained with Hoechst 33342 dye. With a confocal laser scanning microscope, we found that CTX-eGFP was localized mostly in the membrane of BmN cells (Fig. 4).

Decreased melanization of *B. mori* hemolymph by CTX expression. To determine if CTX products affect melanization in hemolymph, we selected 3 types of insects, *B. mori* larvae and pupae and *Eri* silkworm pupae. Melanization was reduced in the hemolymph from insects infected with various viruses compared with untreated insects (4 days post-infection, $p < 0.05$) (Fig. 5, A, B and C). Additionally, we assayed the effects of recombinant *ctx* gene expression from BmNPVs on melanization inhibition *in vitro*. However, a difference in melanization inhibition was not observed when the lysate of BmN cells infected with the three viruses (BmpFB1-*ie1-ctx*, BmpFB1-*ph-ctx* and wild

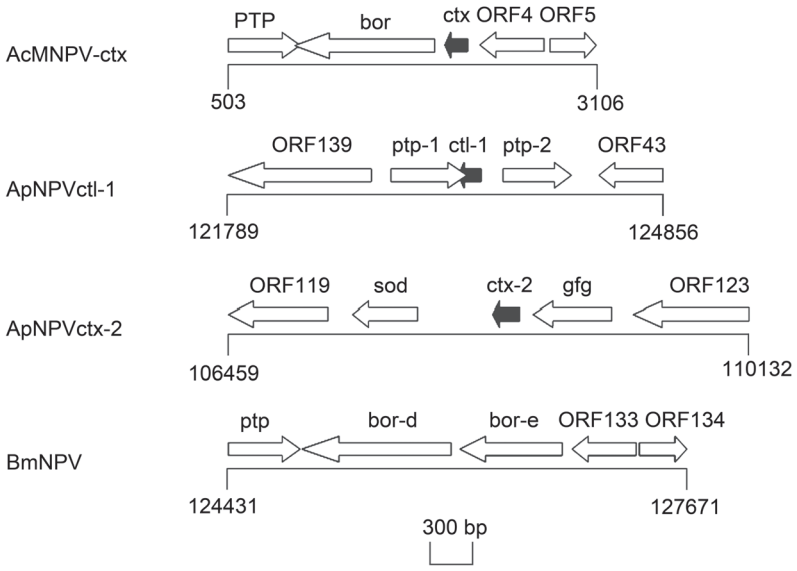

Fig. 3. Analysis of the genomic organization of *ctx* region.

The location of the *ctx* gene according to the nucleotide sequence of AcMNPV, ApNPV and BmNPV in base pairs relative to the start codon of the polyhedrin genes. Arrows indicate ORFs along with the size and direction of transcription. The genes surrounding *ctx* are shown above the arrows. Black arrows indicate the *ctx* gene. The *ctx* gene was not detected in BmNPV.

type BmNPV) was mixed with *B. mori* hemolymph of larvae *in vitro*, respectively. The data indicated that the CTX polypeptide might not prevent hemolymph melanization *in vitro*.

Discussion

The *ctx* gene was initially found in AcMNPV with a conserved structure similar to omega-conotoxins from cone snails (Eldridge *et al*., 1992). Despite substantial homology between BmNPV and AcMNPV, we did not detect the *ctx* sequence in the BmNPV genome. To verify if this gene is unique to AcMNPV, we searched for this CTX peptide sequence within other baculoviruses. Forty analogous sequences were found in the GenBank database, with 28 sequences coming from baculoviruses. Furthermore, 6 baculoviruses have two or more copies of this structure-like protein. Protein structure analysis was performed on the CTX sequences from baculoviruses. We uncovered a conserved cysteine motif structure in these sequences, and the majority contained an N-terminal signal peptide. These results suggest that this gene functions in viral infection.

Phenoloxidases (POs) are present as zymogens in insect hemolymph, and they are involved in melanotic

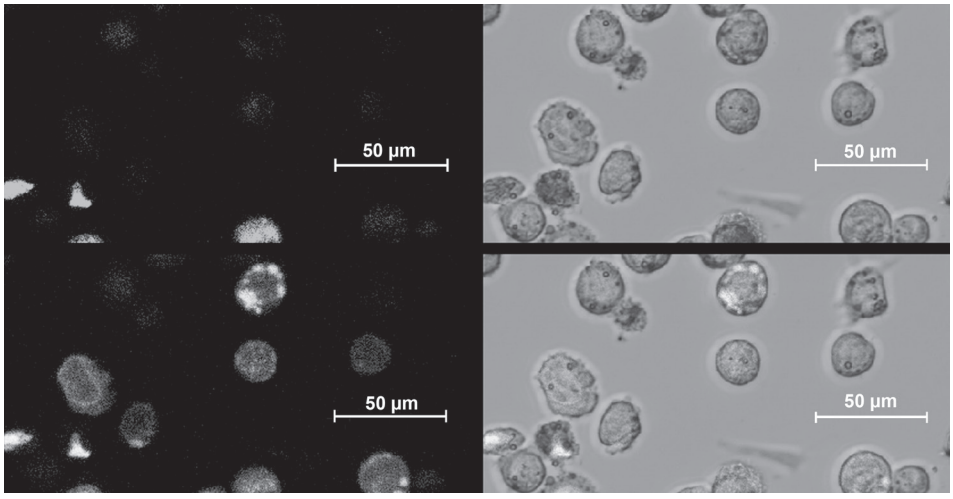

Fig. 4. Subcellular localization of CTX-eGFP fusion protein in BmN cells.

BmN cells were infected with a recombinant BmNPV expressing a ctx-eGFP fusion gene. Using a confocal laser scanning microscope, green fluorescence was mostly observed in the membrane of BmN cells.

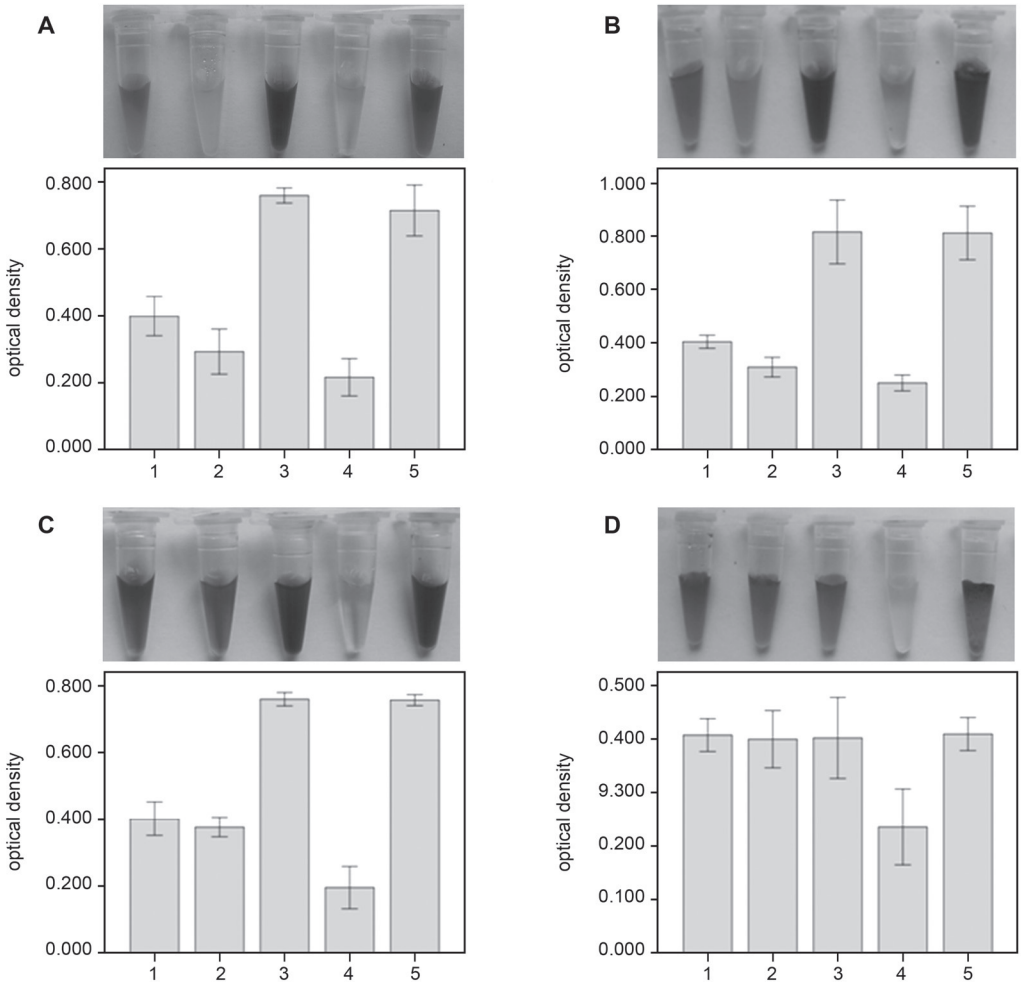

Fig. 5. Analysis of hemolymph melanization in insect larvae and pupae.

Four varieties of treated hemolymph were examined and marked A, B, C, and D. "A" represents hemolymph from infected *B. mori* larvae; "B" represents hemolymph from infected *B. mori* pupae; "C" represents hemolymph from infected *Eri* silkworm pupae; "D" represents hemolymph from *B. mori* larvae *in vitro* mixed with various lysates from BmN cells infected with recombinant BmNPVs. In each hemolymph experiment, 5 treatments were compared. "1" represents the BmpFB1-*ie1*-*ctx* infected hemolymph; "2" represents BmpFB1-*ph*-*ctx* infected hemolymph; "3" represents BmNPV (negative control) infected hemolymph; "4" represents medium saturated with the melanization inhibitor phenylthiocarbamide (PTC) (*i.e.*, the positive control); and "5" represents hemolymph without treatment (blank control).

encapsulation, wound healing and hemolymph coagulation as part of the innate immune response (Theopold *et al*., 2004). POs can generate quinones and other reactive intermediates, which leads to the synthesis of melanin. Melanin is deposited on foreign surfaces to help kill invading pathogens and parasites. This regulation is essential because the products of PO activity are potentially toxic to the host (Lavine and Strand, 2002; Sugumaran, 2002). Some PO inhibitors (POIs) contain identical structures ($CX_3GX_2CX_5CCX_3CX_6C$) (Lu and Jiang, 2007). Because CTX has a conserved cysteine motif structure that is observed in POIs, we predicted that CTX has a function as POI. Phenylthiocarbamide (PTC) is an insect PO inhibitor that prevents hemolymph melanization. PTC is a metal ion chelator that interacts with copper ions from the protease cofactor of tyrosine. It is an effective inhibitor of tyrosine protease activity, and its presence can impair or halt the enzymatic reaction. Thus, we used PTC as a control (Luna-Acosta *et al*., 2011).

We noticed that melanization in *Eri* silkworm hemolymph is more rapid compared with *B. mori* hemolymph when the liquid was exposed to air. Furthermore, hemolymph melanization is faster in pupae compared with larvae (data not shown). Therefore, the hemolymph of *Eri* silkworm pupae was selected for further assessment. We constructed a recombinant BmNPV expressing the AcNPV *ctx* gene and evaluated melanization after infection. Our data imply that the CTX polypeptide downregulates PO activity. When the cell lysate with *ctx* gene products was compounded with the *B. mori* pupa hemolymph *in vitro*, melanization was not obviously decreased compared with the control. These results suggest that the CTX products do not function over a short

time period. The speed of hemolymph melanization varies among Lepidopterae insects, but it is relatively faster in the *Eri* silkworm. ScrNPV, which contains two *ctx*-like genes, can infect *Eri* silkworm larvae. This finding explains why the virus requires two *ctx* genes to prevent hemolymph melanization. However, the specific molecular mechanism is unclear. Further investigation into molecular interaction with PO is required, especially with regard to the actions of the two *ctx*-like genes during baculoviral infection.

Acknowledgements

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