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Deletion of Superoxide Dismutase Gene of *Bombyx mori* Nuclear Polyhedrosis Virus Affects Viral DNA Replication

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Superoxide dismutase (SOD) is an important enzyme which catalyzes superoxide radicals to hydrogen peroxide. A Cu, Zn *sod*-like gene was found in *Bombyx mori* nuclear polyhedrosis virus encoding 151 amino acids. To demonstrate its function, a recombinant virus named *dsBm*NPV with deleted *sod* gene was constructed. It was discovered that the *sod* gene was not essential for viral replication. Studies on growth of budded virus in BmN cells and superoxide dismutase and catalase activities *in vivo* after dsBmNPV infection showed that the titer of dsBmNPV decreased obviously comparing to wild type BmNPV, the *sod* gene was effective on genomic DNA replication of baculovirus, the peak of SOD activity of silkworm infected with wt-BmNPV appeared between 36 and 48 hrs post infection, and with dsBmNPV, it did not appear. And the changes of CAT activity after infection were similar to SOD activity.

Key words: *Bombyx mori,* Superoxide dismutase, BmNPV

Introduction

Baculoviruses have specific host range in insects and cause fatal disease. More than 800 strains of baculoviruses have been isolated and some of them have been used as bio-pesticide to control insect populations in agriculture and forestry. In many cases, NPVs act as a useful tool to express genes of interest by infecting cells and larvae of insects (Lu, 1998). There are some typical represents for research in baculoviruses, such as *Autographa californica* nuclear polyhedrosis virus (AcNPV), *Bombyx mori* NPV, and *Lymantria dispar* NPV. The NPVs have circular, double-strand DNA genomes which encode about 100 genes. Full length genomic DNAs of some NPVs have been sequenced (Areys *et al.*, 1994; Gomi *et al.*, 1999) and also a part of genes were identified. However, more of them were still unknown.

A superoxide dismutase (sod) like gene was cloned and *sod* activity was determined *in vitro* (Wang *et al.*, 1999). The superoxide radical can act both as a reductant and an oxidant to impair DNAs and proteins. SODs and catalase have been showed that they have protective roles in preventing damage from dioxygen metabolites in a cooperation manner. Approach to its function in virus replication process in vivo, a *sod*-deleted BmNPV strain was constructed. Some characters were observed comparing to that of wild type BmNPV.

Materials and Methods

Cell culture

Bombyx mori cells were cultured at 27°C in TC-100 media with 10% FBS. The cells served as the *in vitro* host for propagating and tittering virus.

Construction of transfer vector for deletion of *sod* gene

Genomic DNAs of BmNPV were purified as previously described. After purification, DNAs were digested with *Cla* I at 37°C for 5 hrs. The target fragment with 3,890 base pairs (bp) was separated by agarose gel electrophoresis. The fragment was cloned into vector and was identified. Then the fragment was digested with *Xho* I, and the fragments with 800 bp and 2,500 bp were separated and served as flanking 5' and 3' homological recombinant regions, respectively (Fig. 1). The *lac* Z report gene fused

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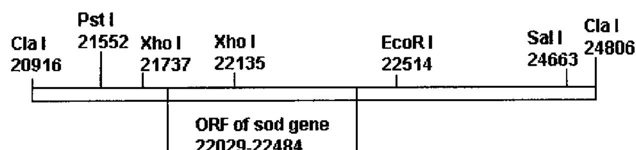

Fig. 1. The fragment of *Bm*NPV *Cla* I (3,890 bp). This fragment was used to construct a transfer vector for selection of *sod*-deleted *Bm*NPV. The report gene of *lac Z* containing eukaryotic promoter was inserted into the *Xho*I region of *sod* open reading frame. The numbers under the restriction enzymes indicate the enzyme site on the genomic DNA of BmNPV.

with SV40 promoter in a length of 3,300 bp was inserted instead of the *Xho* I fragent. The plasmid for replacement of the viral *sod* gene was constructed.

Cotransfection and identification of *sod* deleted BNV strain

Bm cells (2 x 10^4) were seeded into 35 mm dish and incubated for 2 hrs. The cells were washed with TC-100 media (Invitrogen), and 1 ml fresh TC-100 media (serum free) were added to the dish. One μg of BmNPV DNA and 3 μg of the recombinant plasmid were mixed with transfection reagent (Invitrogen). The mixture was added to the medium in a cell culture dish. The dish was placed under 28°C for 4 to 6 days (Summers *et al.*, 1987). The cell was checked with an inverted microscope for signs of infection. When positive signs of infection appeared, the supernatant was diluted for plaque assay. The recombinant virus was selected by screening.

Blot analysis

Purification was completed 3 times by screening for the white plaques. The putative recombinant plaques were placed each in a separate well. Cells infected by NPVs in 4 days were lysed by adding 200 μl of 0.5 mol/l NaOH. The solution was neutralized with 20 μl of 10 mol/l NH_4 acetate. The lysates from each well were spotted onto nitrocellulose filters in a dot blot apparatus. The cross-linked filters were ready for hybridization. The deleted fragment of *sod* gene in a length of 400 bp and the fragment of *lac* Z gene were used as probes to hybridize respectively.

Replication curve of NPVs in infected Bm cells

The Bm cells were infected with *sod*-deleted BmNPV and wild type BmNPV, respectively. The multiplicity of infection (MOI) was one. After incubated with budded viruses for one hour, the supernatant was removed and 2 ml fresh media were added to each dish and this time point was set as the starting point for infection. The supernatant was collected at different time point to calculate the titer of the budded virus.

Superoxide dismutase and catalase activity assays

Fifth instar silkworm larvae were used for infection with *sod*-deleted BmNPV and wild type BmNPV. The blood of 6 larvae was collected for each sample at different time points with three repeats. Superoxide dismutase activity was determined according to the methods described by Shi (1999). Catalase activity was determined according to the methods described by Li (1994).

Results

Identification of transfer vector and recombinant virus for *sod* mutant virus

The transfer vector containing 5-terminal fragment of *sod*

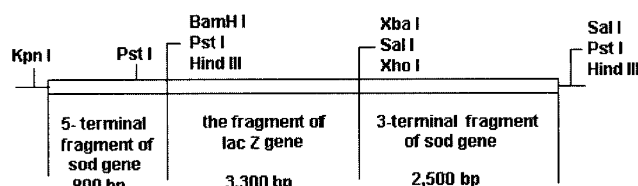

Fig. 2. Construction of transfer vector for deletion of the *sod* gene of BmNPV.

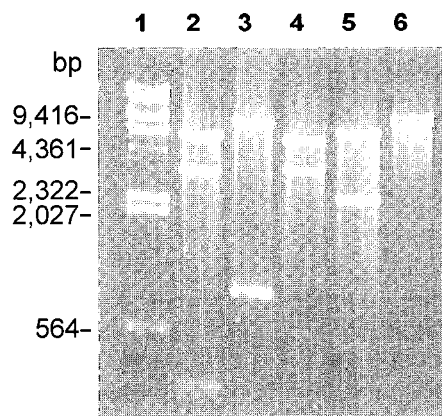

Fig. 3. Identification of recombinant transfer vector. DNA of the recombinant vector was extracted and digested with restriction enzymes. 1, DNA marker (DNA/ *Hind* III); 2, *Pst*I; 3, *Kpn*I/ *BamH* I; 4, *BamHI*/*Xba*I; 5, *Sal*I; 6, *Kpn*I. In lane 2, a 200 bp 5-terminal fragment and a 3,300 bp fragment (600 bp 5-terminal fragment and 2,700 bp vector) were obtained by *Pst*I. In lane 3, a 800 bp 5-terminal fragment was obtained by *BamHI* and *Kpn*I. In lane 4, the fragment of *lac* Z gene was obtained by *BamHI*/*Xba*I. In lane 5, the 3-terminal fragment was obtained by *Sal*I. In lane 6, there is one band since there is only one *Kpn*I site on the full length DNA of the recombinant vector.

gene, the fragment of SV40 promoter-*lac* Z, and 3-terminal fragment of *sod* gene were constructed with about 9.3 kb (Fig. 2). The restriction enzymes were used to identify each fragment inserted into the vector. The 5-terminal fragment of *sod* gene with *Kpn*I and *BamH*I, fragment of SV40 promoter- *lac* Z gene with *BamH*I and *Xba*I, and the 3-terminal fragment of *sod* gene with *Xho*I and *Hind*III were identified on each end by digestion with relevant enzymes, respectively (Fig. 3).

The recombinant virus was obtained by co-transfecting the transfer vector DNA and wild type virus DNA into BmN cells and screening for blue plaques in the presence of X-gal. The virus strains with blue plague were isolated and selected. Then the viral DNAs were extracted for dot blot assay. The result showed that there was positive signal using the *lac* Z gene fragment as probe and negative signal using the *sod*-deleted *Xho*I fragment as probe (Fig. 4).

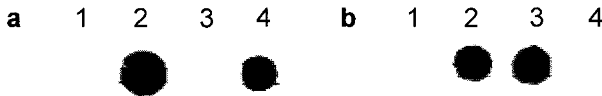

Fig. 4. Dot-blot assay for detection of the fragments of *lac* Z gene and *sod* gene. a, the fragment of *lac* Z gene was detected by *lac* Z DNA as probe. 1, DNA of Bm cells (negative control); 2, the plasmid DNA containing *lac* Z fragment (positive control); 3, genomic DNA of wild type BmNPV; 4, genomic DNA of *sod*-deleted BmNPV. b, the 400 bp *Xho*I fragment of *sod* gene was detected by the 400 bp *Xho*I fragment as probe. 1, DNA of Bm cells (negative control); 2, the plasmid DNA containing the 400 bp *Xho*I fragment of *sod* gene (positive control); 3, genomic DNA of wild type BmNPV; 4, genomic DNA of *sod*-deleted BmNPV. The result indicated the *lac* Z fragment was inserted into BmNPV genome instead of the 400 bp *Xho*I *fragment* of *sod* gene.

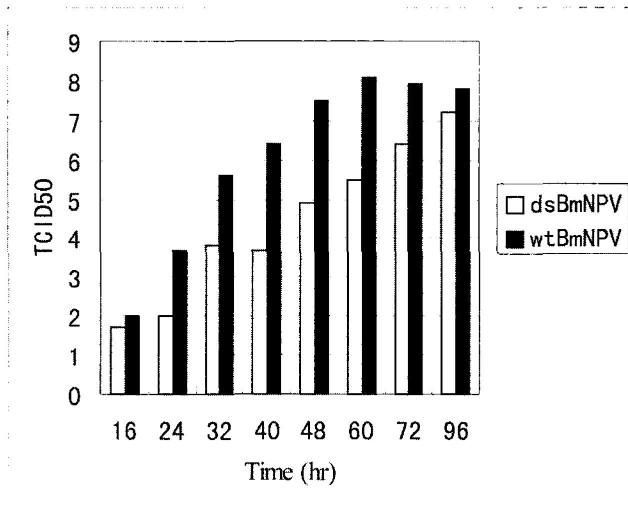

Fig. 5. Budded virus yield in *Bm*N cells at different infected time points. In early stage of infection, the titer of *sod*-deleted *Bm*NPV was obviously lower than that of wild-type *Bm*NPV. The data indicated that the *sod* gene affected DNA replication of *Bm*NPV.

Budded virus yield after infection

The titer of budded virus (BV) increased after infection with wild type BmNPV. There was a peak appearing within 60 to 72 hrs post infection. After that, the titer decreased. When infected with *sod*-deleted BmNPV, at early stage (from 0 to 60 hrs post infection), the titer of budded virus was apparently lower than that of wild type BmNPV (Fig. 5). This indicated that expression of *sod* gene was related to virus replication.

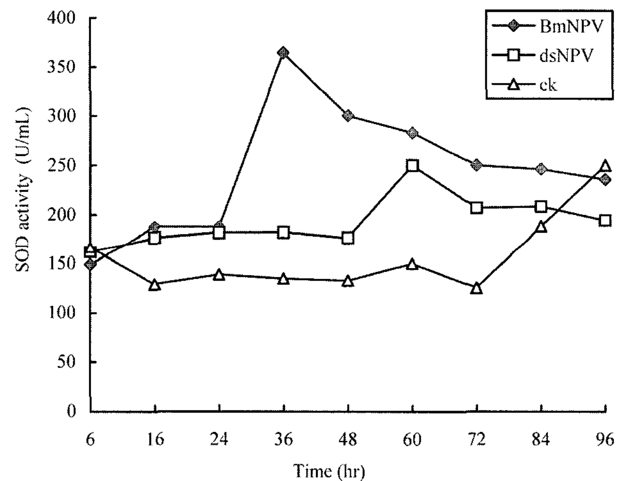

Fig. 6. SOD activity was determined in the blood of *Bombyx mori* larvae at different infected time points.

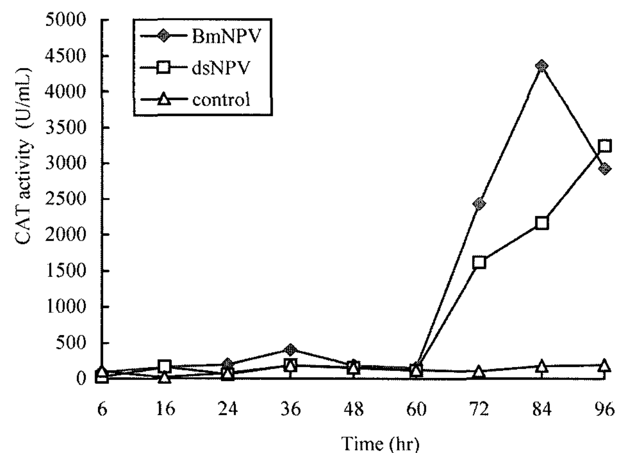

Fig. 7. Catalase activity was determined in the blood of *Bombyx mori* larvae at different infected time points.

Superoxide dismutase and catalase activity in infected silkworm larvae

Peaks of the enzyme activity appeared at 36 hrs point post infection in infected silkworm larvae with wild type virus but not in the cells infected with *sod*-delected virus (Fig. 6). It demonstrated that the viral *sod* gene is active *in vivo*. Higher SOD activity appeared in infected silkworm larvae compared to that in non-infected silkworm larvae during viral infection period. Catalase activity was increased in infected silkworm larvae. Similar to SOD activity, catalase activity in silkworm larvae infected with wild type virus was higher than that treated with *sod*-deleted virus (Fig. 7).

Discussion

In previous studies, the genes, *ie1*, *p143*, *lef1*, *lef2* and *lef3* of baculovirus were identified to be essential for DNA replication. A sequence similar to eukaryotic Cu/Zn *sod* genes were found both in the genome of AcNPV and BmNPV. The *sod* gene of BmNPV was cloned and expressed in bacteria expression system (Wang *et al.*, 1999). The products of *sod* gene were identified to have SOD activity *in vitro*. Being aware of its function *in vivo*, we constraucted the *sod*-deleted BmNPV. The result indicated that the *sod* gene is not necessary during the infection cycle. However, expression of the *sod* gene affects viral DNA replication. Interestingly, catalase gene was no found in the genome of baculovirus. It might be that catalase exists in insect cells and has very high activity.

References

Areys, M. D. (1994) The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* **202**, 586-605.

Fridovich, I. (1989) Superoxide dismutases. *J. Biol. Chem.* **264**, 7761-7764.

Gomi, S. (1999) Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. *J. Gen. Virol.* **80**, 1323-1337.

Li, Z. Z. (1994) A study on the activities of endogenous enzymes of protective system in some insects. *Acta Entomo. Sin.* **37**, 399-403.

Lu, H. S. (1998) Molecular biology of insect viruses. pp. 547-606. China Agricultural Scientech Press, Beijing.

Shi, H. J. (1999) Cloning, sequencing and expression of human copper, zinc-superoxide dismutase cDNA. *Acta. Biochim. Biophys. Sin.* **31**, 6-18.

Summers, M. D. and G. E. Smith (1987) A manual for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station BulletinNo. 1555.

Wang, W. B. (1999) Sequence analysis of *sod* gene of *Bombyx mori* nuclear polyhedrosis virus and its expression in *E. coli*. *Acta. Biochim. Biophys. Sin.* **31**, 405-408.