

Functional expression of lepidopteran-selective neurotoxin in baculovirus: Potential for effective pest management

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Abstract

Recombinant baculovirus expressing insect-selective neurotoxins derived from venomous animals are considered as an attractive alternative to chemical insecticides for efficient insect control agents. Recently we identified and characterized a novel lepidopteran-selective toxin, *Buthus tamulus* insect-selective toxin (ButaIT), having 37 amino acids and eight half cysteine residues from the venom of the South Indian red scorpion, *Mesobuthus tamulus*. The synthetic toxin gene containing the ButaIT sequence in frame to the bombyxin signal sequence was engineered into a polyhedrin positive *Autographa californica* nuclear polyhedrosis virus (AcMNPV) genome under the control of the p10 promoter. Toxin expression in the haemolymph of infected larvae of *Heliothis virescens* and also in an insect cell culture system was confirmed by western blot analysis using antibody raised against the GST-ButaIT fusion protein. The recombinant NPV (ButaIT-NPV) showed enhanced insecticidal activity on the larvae of *Heliothis virescens* as evidenced by a significant reduction in median survival time (ST₅₀) and also a greater reduction in feeding damage as compared to the wild-type AcMNPV.

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Keywords: Baculovirus; Lepidopteran-selective toxin; Recombinant biopesticide; *Heliothis virescens*; *Mesobuthus tamulus*; Scorpion venom; Integrated pest management

1. Introduction

Baculoviruses (nuclear polyhedrosis virus; NPV) are arthropod specific viruses that have long been used as natural insect biological control agents for protection of numerous crop plants [1]. NPVs are major insect pathogens characterized by a large circular double stranded DNA and enveloped rod shaped virions. They provide an attractive alternative to chemical insecticides for controlling insect pests and can be used as efficient vectors for the expression of foreign genes [2–5]. Since NPVs have an effective host range restricted to only a few closely related species, they can be readily integrated into

integrated pest management (IPM) programs without disrupting the balance of beneficial insects and non-target organisms [6–9]. Among different NPVs, the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) is known to infect approximately 39 species of Lepidoptera including serious pests in the genera *Heliothis*, *Spodoptera* and *Trichoplusia* [1]. However, the use of baculoviruses as insect control agents has met with limited success due to lack of field persistence and slower speed of action on insect mortality when compared to classical chemical insecticides [10]. In order to enhance the efficiency of NPVs either by reducing the time to kill host insects or to reduce their feeding capacity, different recombinant baculoviruses have been constructed expressing insect-selective toxins [11–16], hormones [3,17], enzymes involved in insect metabolism [5,10], the maize mitochondrial protein URF13 [18], and by deletion of the *egt* gene [19] which significantly increased the speed of host incapacitation. Regev et al. have generated a recombinant AcMNPV expressing both excitatory LqhIT1 and

Abbreviations: Buta-IT, *Buthus tamulus* insect-selective toxin; AcMNPV, *Autographa californica* Nuclear polyhedrosis virus; GST, Glutathione S-transferase; LD₅₀, Median lethal dose; ST₅₀, Median survival time

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depressant LqhIT2 under the very late p10 and polh promoters respectively, which resulted in synergistic effects with marked reduction in ET₅₀ [20]. In addition to these, several other strategies for the construction of recombinant biopesticides and their potential for pest control have been reviewed recently [21]. Baculovirus expressing insect-selective toxins that affect the ion-channels of excitable cells of insects have proved to be highly promising in increasing the viral efficiency in insect pest control [22]. Continued efforts are being made to identify natural insect-selective peptide toxins derived from venomous animals that can be used for the development of genetically engineered insect-selective baculoviruses with enhanced pesticidal and pestistatic properties targeting selected insect pest populations. In our recent studies, we have reported the purification and characterization of a novel short lepidopteran-selective toxin (*Buthus tamulus* insect-selective toxin; ButaIT) from the venom of the South Indian red scorpion, *Mesobuthus tamulus*, which selectively caused flaccid paralysis in *Heliothis virescens*, but was non-toxic to blowfly larvae and mice (SWISS-PROT PDB Accession number P81761) [23]. This ButaIT is a single polypeptide chain of 37 amino acids cross-linked by four disulfide bridges and showing a high degree of sequence similarity with other short insect toxins such as peptide I [24], Lqh-8/6 [25], neurotoxin P2 [26], chlorotoxin [27], insectotoxin I5A [28], insect toxin I₅ [29] and insectotoxin II [30]. In the present report we describe the construction of recombinant baculovirus expressing ButaIT under the control of p10 promoter targeting lepidopteran larvae, which are major pests of agriculture worldwide.

2. Materials and methods

2.1. Cell lines

Cells of *Spodoptera frugiperda* (Sf-21) were maintained in Excell 401 medium (JRH Biosciences) supplemented with 3% heat inactivated fetal calf serum (Gibco BRL) and 1% penicillin-streptomycin. High Five cells derived from *Trichoplusia ni* (Tn; High Five; Invitrogen) were maintained in ESF 921 medium (Expression Systems, Woodland, CA) supplemented with 1% penicillin-streptomycin (Sigma Chemical Co., USA). The continuous Sf-21 cell line was used for cotransfection, plaque assays and viral (wild-type NPV and recombinant ButaIT-NPV) propagation and the *T. ni* cell line was used for toxin expression.

2.2. Construction of recombinant virus

A toxin gene encoding ButaIT behind a secretory signal sequence from the neuropeptide bombyxin [11,31] was synthesized from synthetic oligonucleotides using baculovirus preference codons for the toxin's amino acid sequence (Fig. 1). The toxin gene was PCR amplified using *pfu* DNA polymerase and the blunt-ended PCR product was cloned into the pCR Script Amp SK (+) cloning vector at the *SrfI* site between the *SacI* (755) and *KpnI* (657) sites using a pCR Script cloning kit and following the manufacturer's instructions (Stratagene). This construct was transformed into competent cells of *E. coli* (XL1 Blue MRF). After confirming the sequence of the toxin gene, including signal sequence and restriction sites, the gene was subcloned into the baculovirus transfer vector, pAcUW21 (Pharmingen) at the unique *BglII* site under the control of the p10 promoter. The recombinant transfer vector was transformed in the XL1Blue MRF strain of *E. coli*. Two clones of the recombinant transfer vector (pAcUW21-ButaIT) carrying the ButaIT gene having a correct orientation and nucleotide sequence were isolated and used for cotransfection into baculovirus (BacPAK6; Clontech) (Fig. 1). Digestions, ligation, transformation and plasmid purification were carried out using standard techniques [32].

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          Bam H I   Bgl II   Eco R I   M (I) K I
5' - AGA TCA GGA TCC AGA TCT GAA TTC ATG AAG ATA
      L L A I A L M L S T V M W
CTC CTT GCT ATT GCA TTA ATG TTG TCA ACA GTA ATG TGG
      V S T R C G P C F T T D
GTG TCA ACA CGC TGC GGC CCC TGC TTT ACC ACC GAC
      P Q A T Q A K C S E C C G
CCC CAA ACC CAA GCC AAA TGC AGC GAG TGC TGC GGC
      R K G G V C K G P Q C I
CGC AAA GGC GGC GTG TGC AAA GGC CCC CAA TGC ATC
      C G I Q * Sac I Bgl II Bam H I
TGC GGC ATC CAA TAAG GAG CTC AGA TCT GGA TTC
TCA GGC - 3'

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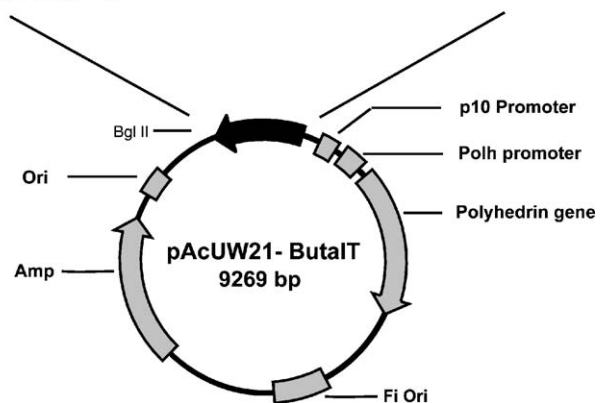


Fig. 1. Construction of recombinant baculovirus transfer vector pAcUW21-ButaIT. Nucleotide sequence of synthetic toxin gene (bold letters) constructed for 37-amino acid sequence of the lepidopteran-selective toxin from *Mesobuthus tamulus* (ButaIT) containing bombyxin signal sequence (underlined) to the 5' end of ButaIT coding sequence. The ButaIT gene was ligated into the *BglII* cloning site of the transfer vector under the control of p10 promoter.

2.3. Co-transfection of ButaIT into baculovirus

The recombinant transfer vector pAcUW21-ButaIT and *Bsu36* I digested polyhedrin positive BacPAK6 (AcMNPV) viral DNA (Clontech) were cotransfected into Sf-21 cells using the lipofectin mediated transfection (Life Technologies) method as described by O'Reilly et al. [33].

2.4. Isolation of recombinant NPV

The ButaIT-NPV was purified from cotransfected Sf-21 cells forming polyhedrin positive plaques. The individual plaques were purified in three successive rounds and the presence of insert was verified by PCR analysis using ButaIT gene specific primers and the resultant pure recombinant virus was amplified in vitro using Sf-21 cells and the aliquots of virus were stored at -80°C until further use.

2.5. Construction of GST-ButaIT fusion protein

The ButaIT toxin gene excluding the bombyxin signal sequence was amplified using PCR primers (primer 1: 5' CGGAATTCGCTGCGGCCCTGCTTTAC; PRIMER 2: 5' GAGTTCGACTTATTGGATGCCGCAGATG-CATTG) having *EcoRI* and *SalI* sites (underlined sequences) at the 5' and 3' end respectively. The resultant ButaIT gene PCR product was cloned into GST-fusion protein vector pGEX-6P-1 (Pharmacia Biotech) at the multiple cloning site located at the C-terminus of GST between the *EcoRI* and *SalI* sites. The pGEX-6P1-ButaIT plasmid was transformed into BL-21 strain of *E. coli*. After confirming the positive clones using sequence analysis, the ButaIT toxin gene (37 amino acids) was expressed as a GST-ButaIT fusion protein and affinity purified using glutathione sepharose 4B as per the protocol of Pharmacia

Biotech. The affinity purified GST-ButaIT fusion protein was run on SDS-PAGE and the fusion protein was used to raise polyclonal antibodies (Fig. 2). SDS-PAGE was conducted on 1 mm thick slab gels consisting of a 10% acrylamide gel at pH 8.8 and 4% acrylamide gel at pH 6.8 in the presence of 0.1% of sodium dodecyl sulphate [34]. The samples were denatured with 1% SDS and 1% β -mercaptoethanol at 100 °C for 2 min. Proteins were stained with 0.1% Coomassie Brilliant Blue R-250.

2.6. Immunoblot analysis

Third instar larvae of *H. virescens* were orally infected with polyhedrin inclusion bodies (PIB) containing recombinant polyhedrin-positive ButaIT-NPV or polyhedrin positive wild-type NPV. Diet plugs were inoculated with 3000 PIBs of either recombinant or wild-type NPV. Larvae were allowed to consume the diet plugs and upon onset of symptoms of flaccid paralysis (76 h), the haemolymph from both recombinant and wild-type NPV infected larvae was collected. Haemolymph was collected from an incision in the larval proleg, then immediately frozen in a dry ice/ethanol bath and stored at –80 °C.

Similarly *T. ni* H5 cells were infected with either recombinant or wild-type budded virus at a multiplicity of infection of 1. Samples (5 ml) were collected every day and cells were harvested by centrifugation (400 \times g for 10 min). The pellet was suspended in 3 ml Tris/HCl buffer 50 mM, pH 8.0 containing PMSF (phenylmethyl sulfonyl fluoride), EDTA and DTT. The cells were disrupted using an ultrasonic homogenizer and centrifuged at 400 \times g for 10 min. The supernatant was stored at –20 °C until further analysis. The culture medium was collected and the secreted toxin was precipitated with acetone. After centrifugation the precipitated toxin was stored at –20 °C.

Western blot analysis of the above samples was performed for the detection of in vivo and in vitro toxin expression. The samples were mixed with 0.5 volumes of SDS-PAGE buffer and analyzed by SDS gel electrophoresis in 10–20% gels (Novex) following the manufacturer's instructions. Gels were electroblotted on Nitro Bind nitrocellulose membrane (0.2 μ m pore size) (Micron Separations Inc., USA) and immunostained with polyclonal antibodies raised against GST-ButaIT fusion protein, followed by application of HRP

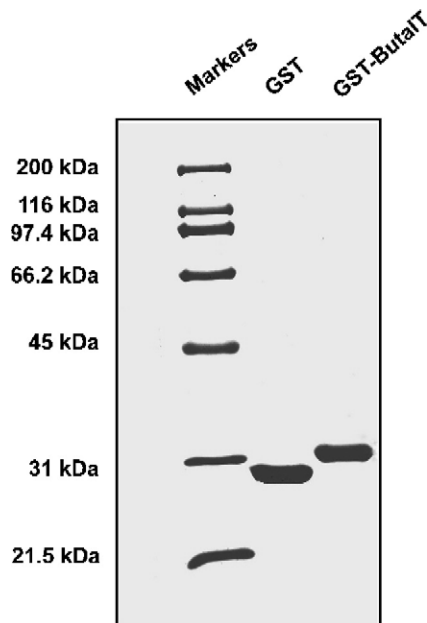


Fig. 2. SDS-polyacrylamide gel electrophoresis of GST and GST-ButaIT fusion protein expressed in BL21 strain of *E. coli* and purified from bacterial lysates by affinity chromatography using glutathione sepharose 4B. Lane 1: molecular weight markers (Myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor 21.5 kDa); Lane 2: GST; Lane 3: GST-ButaIT fusion protein. 20 μ g of protein was stained with Coomassie Brilliant Blue R-250.

conjugated goat-rabbit antibody. Toxin was detected with a DAB kit according to the manufacturer's instructions (Boehringer Mannheim).

2.7. Bioassays

PIBs of both wild-type and recombinant virus used for monitoring median lethal dose (LD_{50}) and median survival time (ST_{50}) were purified from virus infected cadavers of *H. virescens* as described by O'Reilly et al. [33]. The PIBs were counted using a standard hemocytometer, and were diluted in sterile H_2O for making serial dilutions.

2.8. Determination of median survival time (ST_{50})

Neonate larvae of *H. virescens* were infected with 3000-polyhedra/ μ l using the droplet feeding method [35]. Larvae that consumed the virus were maintained in individual containers with excess artificial diet at 26 °C, 16 hL: 8hD cycle. 30 to 40 larvae were used for each treatment and the entire experiment was replicated four times. Mortality was monitored at 4 h intervals until all the larvae in each group showed 100% mortality. The median survival times (ST_{50}) and 95% confidence intervals for the replicates of each virus and also for pooled data were calculated using the Kaplan–Meier non-parametric estimator of survival functions [36,37].

2.9. Determination of median lethal dose (LD_{50})

Tobacco budworm, *H. virescens*, eggs obtained from the United States Department of Agriculture/Agriculture Research Service (Stoneville, MS) were incubated and neonates were placed on artificial diet in 24-well Falcon plates and maintained at 26 °C, 16L: 8D cycle. Newly molted second instar tobacco budworm were individually dosed in 24-well Falcon plates on 1 mm³ diet plugs containing 1 μ l of different doses (10, 25, 50, 100, 200, 500, 1000, 3000, 10000 PIBs) of wild-type-NPV or ButaIT-NPV. Concentrations ranging from LD_{10} to LD_{99} were selected for dose-mortality studies. Larvae that consumed the dose in 24 h were transferred to individual 35 ml diet cups having excessive artificial diet and mortality was observed daily. The median lethal dose and 95% confidence intervals for the replicates of each virus and also for the pooled data was determined using Probit analysis. Dose versus mortality studies were carried out in four replicates with 30–40 larvae per treatment.

The LD_{50} and ST_{50} values were tested for homogeneity among replicates and also between WT-NPV and ButaIT-NPV using Kruskal–Wallis test of significance [38]. All the statistical analyses were performed by using SPSS 11.5.

3. Results

3.1. Construction of recombinant baculovirus encoding ButaIT synthetic gene

A synthetic DNA encoding the *Buthus tamulus* lepidopteran-selective toxin (ButaIT) with a bombyxin signal sequence was successfully constructed by using four complementary oligonucleotide pairs. The bombyxin signal sequence was incorporated at the 5' end of the coding region to facilitate protein secretion (Fig. 1). The blunt ended ButaIT DNA was cloned into PCR script (SK+) cloning vector for PCR amplification and sequence determination. The toxin gene coding sequence was cleaved from the PCR script and was subcloned into the baculovirus transfer vector to produce pAcUW21-ButaIT. The orientation of ButaIT inserts in the vector was confirmed by restriction endonuclease analysis and by nucleotide sequence. Co-transfection of *Spodoptera frugiperda* (SF-21) cells with pAcUW21-ButaIT plasmid and linearized AcMNPV DNA resulted in polyhedrin-positive recombinant AcMNPV (ButaIT-

NPV), which was plaque purified to genetic homogeneity. The recombinant AcMNPV was further amplified to provide stocks of virus (10^7 to 10^8 pfu/ml), which were used for in vitro and in vivo expression of toxin and for bioassays.

3.2. Expression of ButaIT

The expression of ButaIT by the recombinant virus in the infected *T. ni* cells or by the infected *H. virescens* larvae was confirmed by Western blot analysis probed with polyclonal antibodies raised against GST-ButaIT fusion protein (Fig. 2). The presence of ButaIT in the haemolymph sample of 3rd instar *H. virescens* larvae was observed after 72 h post-infection. The apparent molecular weight of ButaIT determined using immunoblot analysis was found to be identical to that of the native mature toxin, thus confirming the correct cleavage of signal sequence from the peptide during its expression (Fig. 3A). The toxin was also detected in the *T. ni* cell culture medium after 72 h post-infection (Fig. 3B). The expression of ButaIT was first detected after 24 h and showed maximum expression after 96 h post-infection in *T. ni* cell extracts (Fig. 3C).

The activity of recombinant ButaIT was monitored by injecting extracts of ButaIT-NPV infected *T. ni* cells or culture

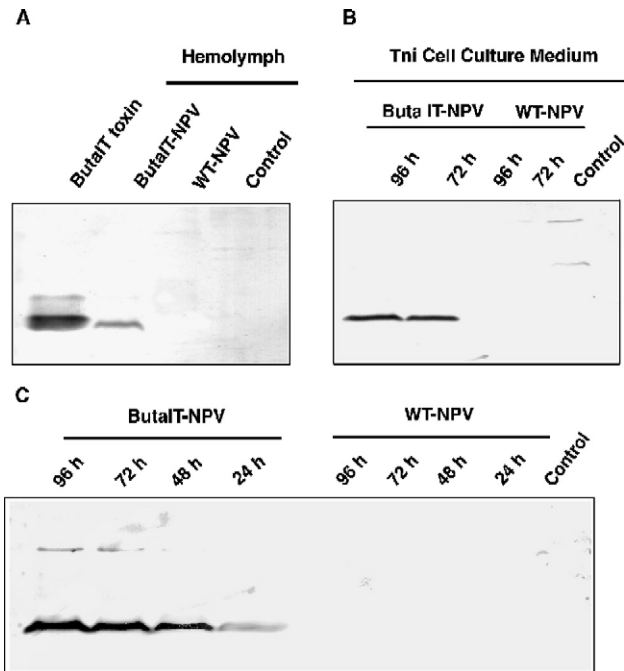


Fig. 3. Immunoblot analysis of ButaIT using antibodies raised against GST-ButaIT fusion protein. (A) Haemolymph samples from infected larvae of *H. virescens*. Lane 1. ButaIT toxin, lane 2. Haemolymph from ButaIT-NPV infected larvae, lane 3. Haemolymph from wild-type NPV infected larvae; lane 4. Haemolymph from uninfected control larvae. (B) Time-course expression of ButaIT in *T. ni* cell culture medium. Lanes 1 and 2—Toxin expression in culture medium after 96 h and 72 h post-infection with ButaIT-NPV; lanes 3 and 4—Culture medium after 96 h and 72 h post-infection with wild-type NPV; Lane 5—*T. ni* cell culture medium of uninfected control. (C) Time-course expression of ButaIT in *T. ni* cell extract: lanes 1 to 4—ButaIT expression in *T. ni* cells after 96, 72, 48 and 24 h post-infection with ButaIT-NPV; lanes 5 to 8—*T. ni* cell extract after 96, 72, 48 and 24 h post-infection with wild-type NPV; lane 9—uninfected *T. ni* cell extracts.

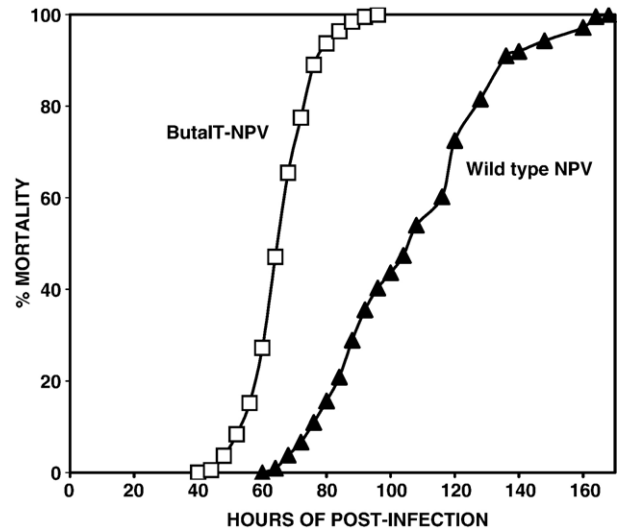


Fig. 4. Survival time analysis of recombinant (ButaIT-NPV) and wild-type NPV viruses on *Heliothis virescens*.

medium into 3rd instar larvae of *H. virescens*. Larvae injected with the extracts of cells collected at 96 hpi (~ 2 – $10 \mu\text{g}$ crude protein/larva) or 1 – $10 \mu\text{l}$ of culture medium (10^7 pfu/ml) showed characteristic flaccid paralysis similar to the purified toxin. None of these symptoms were observed in larvae injected with cell extract or culture media from wild-type NPV.

3.3. Bioassays

Analysis of bioassay indicated that ST_{50} and LD_{50} values were significantly lowered by 42.8% and 28.1% respectively for ButaIT-NPV infected larvae when compared to WT-NPV infected larvae. The homogeneity analysis in each virus treatment was performed using Kruskal–Wallis test and no significant differences were found in ST_{50} ($\chi^2=4.283$; $df=3$; $P=0.232$) and LD_{50} ($\chi^2=3.0$; $df=3$; $P=0.392$) values among replicates. After ensuring that there is no significant difference among replicates, the data from all the replicates were pooled for the determination of ST_{50} and LD_{50} for each virus and also

Table 1

Median lethal dose (LD_{50}) and median survival time (ST_{50}) analysis for wild-type and recombinant NPV on second instar or neonate larvae of *Heliothis virescens*

Virus	ST_{50} (h)		LD_{50} (PIBs)	
	95% CL	\pm SE	(95% CL)	Slope \pm SE
Wild-type NPV	112 (102–122)	5.0	176.4 (125.2–248.4)	1.118 \pm 0.053
ButaIT-NPV	64 (57–71)*	3.5	126.8 (74–221)*	0.846 \pm 0.052
% change over wild-type NPV	–42.8		–28.1	

Bioassays were carried out in quadruplicate; Median survival times (LT_{50}) were determined using Kaplan–Meier Non-parametric estimation; Median lethal dose (LD_{50}) were estimated using Probit analysis; Significance tests were performed using Kruskal–Wallis test; ST: Survival time; LD: Lethal dose; PIB: Polyhedrin inclusion bodies; * Data for ButaIT-NPV were significantly different from wild-type NPV at $P=0.05$.

for the analysis of significance between WT-NPV and ButaIT-NPV. The ButaIT-NPV caused significant reduction in median survival time (Fig. 4) when compared to WT-NPV ($\chi^2=5.398$; $df=1$; $P=0.02$). Similarly, ButaIT-NPV provided a significantly lower LD₅₀ value when compared to WT-NPV ($\chi^2=5.33$; $df=1$; $P=0.02$) (Table 1).

4. Discussion

Reducing time to kill host insects and their feeding capacity are the main goals for improving the effectiveness of baculoviruses [39]. Recombinant baculoviruses containing insect-specific toxin genes or with other modifications for improved speed of action have been designed, developed and tested in various laboratories. Among all the strategies, the insecticidal efficacy of baculoviruses has been enhanced by 30–40% when their speed of kill was compared with baculoviruses expressing anti-insect toxins [12–15] offering an opportunity to improve and augment crop protection strategies.

Although the expression of excitatory toxins such as AaIT has been considered to show high insecticidal efficiency, the reports of Gershburg et al. [16] have shown similar higher insecticidal efficacy of virus expressing depressant toxins on par with excitatory toxins. They have also reported that depressant toxins expressed by AcMNPV are more stable than excitatory toxins and their bioactivity may be more than excitatory toxins. It was also reported that toxin AaIT was found to be more potent on some insects, but was of surprisingly poor activity on lepidopteran larvae [40]. Expression of lepidopteran-selective toxin would be highly advantageous in controlling lepidopteran larvae which attack over 60 crops ranging from maize and sorghum to various legumes such as chick pea and pigeon pea to tomatoes, sunflower and cotton [41]. Hence, we have synthesized a gene encoding lepidopteran-selective depressant toxin derived from the venom of *Mesobuthus tamulus* (ButaIT) and have expressed it using the p10 promoter-based AcMNPV expression system. We have chosen the p10 promoter for expression because the infectivity of recombinant NPV was higher in very late promoters such as p10 when compared to an immediate early promoter [42]. Murphy et al. [43] confirmed that the recombinant virus containing the p10 promoter might be advantageous for the maintenance of the biological activity of recombinant proteins as well as their solubility.

Biological assays suggest that the larvae infected with the wild-type virus were pale and swollen in appearance and lysed rapidly following death of the larvae. Lysis was also observed with the ButaIT-NPV infected larvae, but occurred faster than with the wild-type virus, and infected larvae exhibited depressant neurological symptoms similar to those caused by injection of purified ButaIT from scorpion venom. Temporal expression of ButaIT-NPV has shown a gradual increase of toxin expression both in *T. ni* cells and culture medium. Similar trends were also observed in the haemolymph of infected larvae. The median survival time (ST₅₀) and median lethal doses were significantly decreased with ButaIT-NPV when compared to WT-NPV. It was also observed that ButaIT-NPV infected larvae did not show any knock-off effect as was observed with

AcAaIT-NPV infected larvae [44]. However, both recombinants significantly reduced the feeding damage compared to wild-type NPV. The studies of Simpson [45] showed that mean weight gain of larvae fed with ButaIT-NPV is similar to the larvae fed with AcAaIT-NPV. However, ButaIT-NPV treated larvae showed a one-fold reduction in feeding compared to AaIT-NPV and a six-fold reduction compared to wild-type NPV. Hence, the ButaIT-NPV produced anti-feeding phenomena prior to death, which will lead to a greater reduction in feeding damage than predicted. Contrary to AaIT-NPV, ButaIT-NPV infected larvae showed a massive viral induced tissue damage leading to liquefaction of larvae, which is essential for vertical dispersal of virus that may be desirable for long-term control of insect pests in the environment.

It is also reported that AcAaIT produce significantly fewer progeny polyhedra than WT-NPV and these recombinant viruses may thus possess low ability to develop an epizootic [46]. Cory et al. [47] reported that genetically modified viruses, which express AaIT appear to reduce the secondary cycle of infection, compared to wild-type virus in the field since mean number of polyhedral inclusion bodies was less than that obtained in larvae inoculated with the wild-type viruses. However, the ButaIT-NPV infection surprisingly showed higher productivity of progeny virus on par with wild-type NPV, which competes with wild-type virus in the field for secondary transmission and is also advantageous as microbial agents for long-term control of insect pests.

Our findings represent further improvement in the insecticidal efficiency of baculovirus that expresses lepidopteran-selective toxin, which may offer a selective approach in the control of lepidopteran insects. However, extensive studies have to be conducted to study the potential application of this virus in the control of a variety of lepidopteran species and also to study the adverse effects of this virus, if any, on the non-target organisms. In addition, further testing on the genetic stability of the virus and the likelihood of gene transfer between viruses are planned in order to establish the efficacy of recombinant baculovirus.

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