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Expression of Fusion Protein with *Autographa californica* Nuclear Polyhedrosis Virus Polyhedrin and *Bacillus thuringiensis* cryIA(c) Crystal Protein in Insect Cells

곤충세포주에서 *Autographa californica* 핵다각체병 바이러스의 다각체 단백질과 *Bacillus thuringiensis* cryIA(c) 내독소 단백질의 융합 단백질 발현

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ABSTRACT We have now constructed a novel recombinant baculovirus producing fusion protein with *Autographa californica* nuclear polyhedrosis virus (AcNPV) polyhedrin and *Bacillus thuringiensis* (Bt) cryIA (c) crystal protein. The fusion protein expressed by the recombinant baculovirus in insect cells was characterized. The N-terminal of cryIA (c) gene of Bt subsp. *kurstaki* HD-73 was introduced under the control of polyhedrin gene promoter of AcNPV, by fusion in the front of intact polyhedrin gene or by insertion into the *Hind*III site in polyhedrin gene. The recombinant baculoviruses were named as BtrusI or BtrusII, respectively. Although single transcript from the fusion protein gene was apparently observed, BtrusI was produced the two proteins, 92 kDa fusion protein and only polyhedrin. Inaddition, fusion protein produced by BtrusI did not form polyhedra. Interestingly, however, the cells infected with BtrusII did not show a 33 kDa polyhedrin band as a cells infected with BtrusI. Cells infected with BtrusII were only produced fusion protein, but the polyhedra formed by fusion protein was not observed. To determine the insecticidal toxicity of fusion protein, therefore, Sf9 cells infected with BtrusI were inoculated to *Bombyx mori* larvae. Sf9 cells infected with BtrusI that expressed the fusion protein caused larval mortality although the insecticidal toxicity was low. In conclusion, our results clearly demonstrated that the fusion protein with polyhedrin and Bt cryIA (c) crystal protein have a insecticidal toxicity.

KEY WORDS *Autographa california* nuclear polyhedrosis virus, polyhedrin, *Bacillus thuringiensis*, cryIA (c) crystal protein, insect cells

초 록 *Autographa californica* 핵다각체병 바이러스(AcNPV)의 다각체 단백질과 *Bacillus thuringiensis* (Bt) cryIA (c) 내독소 단백질의 융합단백질을 생산하는 새로운 재조합 바이러스를 제작하고, 곤충세포주 (*Spodoptera frugiperda* 9)에서 발현된 융합단백질의 특성을 분석하였다. Bt *kurstaki* HD-73의 cryIA (c) 내독소 단백질 유전자의 N-말단은 AcNPV 의 완전한 다각체 단백질 유전자의 앞쪽에 융합함에 의하여 또는 다각체 단백질 유전자내의 제한효소 *Hind*III 부위에 삽입함에 의하여 다각체 단백질 유전자의 프로모터 조절하에 도입하였다. 이렇게 작성된 재조합 바이러스를 각각 BtrusI 또는 BtrusII라고 명명하였다. BtrusI은 분명히 단일 전사체를 보임에도 92 kDa의 융합 단백질과 다각체 단백질의 두 단백질을 생산하였다. 또한 BtrusI에 의해 만들어진 융합 단백질은 다각체를 형성하지

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않았다. 한편, BtrusII에 의해 감염된 곤충세포주에서는 33 kDa의 다각체 단백질은 보이지 않았고 단지 융합 단백질만 생산하였으나 다각체는 형성하지 않았다. 따라서 BtrusI에 의해 생산된 융합 단백질의 독성을 조사하기 위하여, BtrusI으로 감염된 곤충세포주를 2령 누에 (*Bombyx mori*)에 접종한 결과 융합 단백질에 의한 독성이 관찰되었다. 결론적으로 다각체 단백질과 Bt cryIA(c) 내독소 단백질에 의한 융합 단백질이 독성을 가지고 있음을 확인하였다.

검색어 *Autographa california* 핵다각체병 바이러스, 다각체단백질, *Bacillus thuringiensis*, cryIA(c) 내독소단백질, 곤충세포주

INTRODUCTION

Baculoviruses have an attractive potential as biological control agents for insect pest management owing to their host specificity, efficacy and stability. However, baculoviruses have a limited host range, and the insecticidal action is relatively slow. The length of the time taken to kill the infected insect is particularly problematic because it may take from several days to weeks before the infected insect dies or at least ceases to damage the crop. Thus, this has limited their use as control agents of insect pests (Granados & Federici, 1986; Maeda, 1995).

Therefore, genetic engineering technology provides a means of improving baculoviruses for use as a specific pest control agent. A number of attempts have been made recently to produce baculoviruses with improved speed to kill, by inserting various genes into the virus genome, under the control of the polyhedrin promoter so that the gene is expressed within the infected cell. Foreign genes that have been introduced for this purpose include those encoding for the *Buthus eupeus* insect toxin-1 (Carbonell *et al.*, 1988), *Manduca sexta* diuretic hormone (Maeda, 1988), *Bacillus thuringiensis* subsp. *kurstaki* HD-73 δ-endotoxin (Merryweather *et al.*, 1990; Martens *et al.*, 1990), *Heliothis virescens* juvenile hormone esterase (Hammock *et al.*, 1990; Bonning *et al.*, 1992; Eldridge *et al.*, 1992), *Pyemotes tritici* TxP-I toxin (Tomalski & Miller, 1991), *Androctonus australis* AaHIT toxin (Stewart *et al.*, 1991; Maeda *et al.*, 1991), and insect-specific spider toxins (Hughes *et al.*, 1997). Actually, among them only those genes for the mite toxin gene and a scorpion toxin showed a significant increase in pathogenicity.

In contrast, *Bacillus thuringiensis* (Bt) δ-endotoxin has been demonstrated to be effective in controlling pest populations. Bt δ-endotoxin accumulates in large amounts during its sporulation forming crystalline occlusions. When ingested by a susceptible insect, the crystal protein is solubilized in the alkaline environment of the gut and cleaved to a smaller active protein by the action of proteases present in the gut juice. The activated toxin causes disruption of the gut, and this leads to cessation of feeding and death of the infected insect in 24～48 hrs. The lepidoptera-specific crystal protein gene (Cry I) encodes 130～140K proteins, which form bipyramidal crystals. The toxicity of crystal protein is greatly enhanced if the protein is cleaved by proteases to a highly active fragment of around 60～70K which corresponds to the N-terminal half of the protein. This active fragment is thought to consist of at least two domains, the toxic and cell-binding domains. It is generally known that typical crystal proteins of Bt are composed of active and structural fragments (Adang *et al.*, 1985; Aronson *et al.*, 1986; Hofte & Whiteley, 1989).

Thus, full-length and truncated forms of crystal protein genes were inserted into the baculovirus genome to enhance the pathogenicity to insect larvae (Merryweather *et al.*, 1990; Martens *et al.*, 1990, 1995; Ribeiro & Crook, 1993). However, no enhancement in pathogenicity of recombinant viruses was observed with insertion of the Bt δ-endotoxin genes even though the toxins alone are highly insecticidal.

The AcNPV polyhedrin gene was fused with N-terminal region of Bt cryIA (c) crystal protein gene in this study because C-terminal region of the gene is not required for toxic activity (Adang *et al.*, 1985; Hofte & Whiteley, 1989). Therefore, here we have now constructed a novel recombinant baculovirus producing fu-

sion protein with AcNPV polyhedrin and Bt cryIA (c) crystal protein. The fusion protein expressed by the recombinant baculovirus in insect cells was characterized.

MATERIALS AND METHODS

Cell Lines and Viruses

Spodoptera frugiperda (Sf9) cells used in this study were maintained at 27°C in TC-100 medium (Sigma) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS, Gibco). Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant virus were propagated in Sf9 cells. The titer of viruses was determined by the plaque assay in Sf9 cells as described by Summers & Smith (1987) and O'Reilly *et al.* (1992).

Construction of Baculovirus Transfer Vector

The *Xho*I-*Sna*BI fragment containing AcNPV polyhedrin gene was inserted into the baculovirus transfer vector pBacPAK8 to yield pBacPAK-AcPol. In the second step, Bt subsp. *kurstaki* HD-73 cryIA(c) crystal protein gene, excised with NdeI from pN6.6 (Adang *et al.*, 1985), was treated with an exonuclease III (Stratagene) to produce 1,833 bp fragment (+388 to +2,221) of its N-terminal region. The fragment was inserted into the *Xho*I site in the front of polyhedrin gene of pBacPAK-AcPol to yield pBtrusI and into the *Hind*III site within the polyhedrin gene of pBacPAK-AcPol to yield pBtrusII (Fig. 1).

Construction of Recombinant AcNPV

The cell culture dish (35-mm diameter) seeded with $1-1.5\times10^6$ Sf9 cells was incubated at 27°C for 1 hr to allow the cells to attach. One microgram of BacPAK6 viral DNA, 5 μg of pBtrusI or pBtrusII DNA in 20 mM HEPES buffer and sterile water were mixed in a polystylene tube to make a total volume of 50 μl. Fifty microliters of 100 μg/ml Lipofectin TM (Gibco) were gently mixed with the DNA solution to be incubated at the room temperature for 30 min. The cells were, after washing twice with 2 ml serum-free TC-100 medium, added with thw same fresh solution (1.5 ml). The Lipofectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubating at 27°C for 5 hr, 1.5 ml TC-100 medium containing antibiotic-antimycotic (Gibco) and 10% FBS was added to each dish for further the incubation. At 5 days postinfection (p.i.), the supernatant was harvested, clarified by centrifugation at 2,000 rpm for 5 min, and stored at 4°C before plaquing on Sf9 cells. To plaque purify recombinant AcNPV, 1.5×10^6 Sf9 cells were seeded per well on a 6-well plate and the supernatant was added to each well.

PCR

Introduction of a fusion gene under the control of the polyhedrin gene promoter was analyzed by PCR from BtrusI or BtrusII genomes by two different primers (5′-ACCATCTCGCAAATAAATAAG-3′ and 5′-GCGA-TCTAAGACACGCAACA-3′) (Clontech). Viral DNA used as a template was purified from the supernatant of Sf9 cells infected with BtrusI or BtrusII. After a 35-cycle amplification (94°C for 1 min; 55°C for 1 min; 72°C for 1 min), PCR products were precipitated with ethanol, centrifuged at 10,000 xg for 30 min, and rinsed with 70% ethanol. These DNAs were analyzed by agarose gel electrophoresis.

Microscopy

Polyhedra were air dried, coated with carbon, and stained with gold to be observed by a scanning electron microscope (Phillips SEM 515). In addition, polyhedra was fixed for 2 hr in 3% glutaraldehyde [in 0.1 M cacodylate buffer (pH 7.4)]. After postfixation in 1% OsO_4 (in the same buffer), the sample was dehydrated in an ethanol-propylene oxide series and embedded in an Epon-Araldite mixture. Thin section was cut with a Sorvall MT-500 ultramicrotome, and examined and photographed in a transmission electron microscope (Phillips CM 20). Light microscopy of Sf9 cells infected with the recombinant virus was performed with an inverted phase contrast microscope (Hund, Wilovert S).

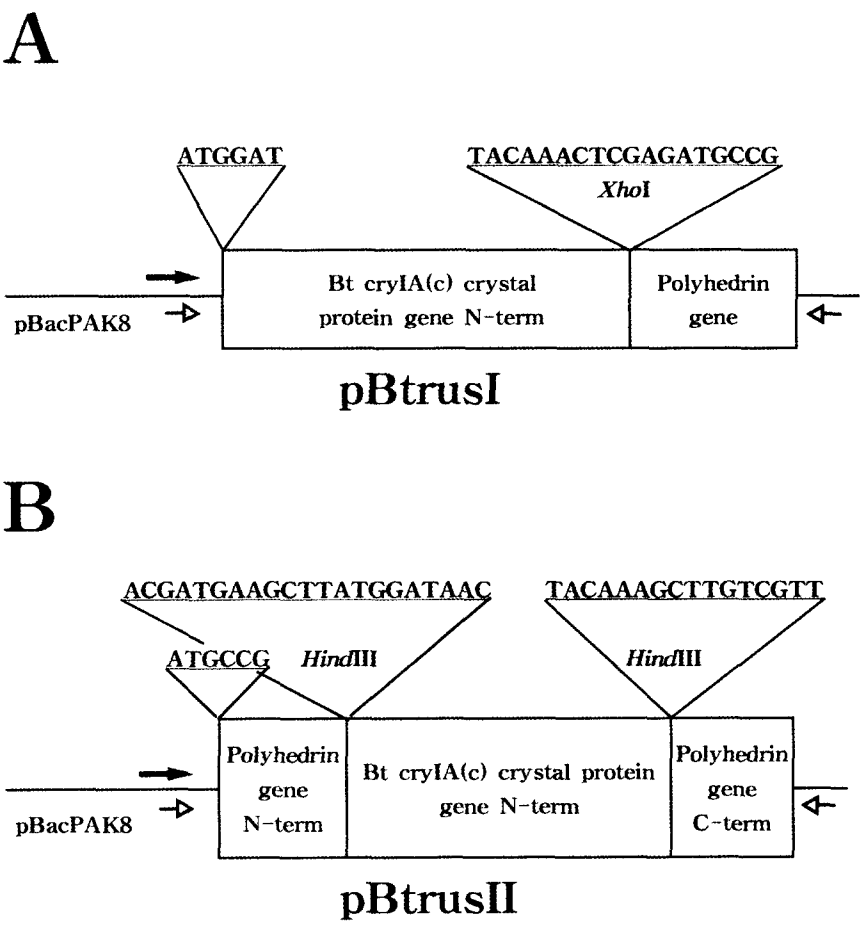

Fig. 1. The baculovirus transfer vector maps used to generate recombinant viruses. N-terminal of the cryIA(c) gene of Bt subsp. *kurstaki* HD-73 was introduced into the genome of the AcNPV, by inserting in the front of the intact polyhedrin gene (pBtrusI) (A) or into the *Hind*III sites within the polyhedrin gene (pBtrusII) (B). The successful introduction of the fusion gene was confirmed by PCR using genomic DNA extracted from the recombinant viruses (C). Solid arrows indicate baculovirus polyhedrin gene promoter and orientation. Open arrows indicate primer positon of PCR.

SDS-PAGE and Western Blot

Sf9 cells were mock infected or infected with the wild-type AcNPV and recombinant AcNPV in a 35-mm diameter dish (1.0×10^6 cells) at a MOI of 5 PFU per cell. After incubation at 27°C, cells were harvested at 1, 2, and 3 days p.i. For SDS-PAGE of cell lysates, Sf9 cells infected with or without viruses were washed twice with the phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) and mixed with the sample buffer (5% SDS, 10% β-mercaptoethanol, 0.02% bromophenol blue, 20% glycerol). Samples were boiled for 5 min and clarified by centrifugation (10,000 xg for 1 min). The total cellular lysates were subjected to 10% SDS-PAGE (Laemmli, 1970), electroblotted and incubated with the anti-AcNPV polyhedrin or anti-crystal protein antibody (Towbin *et al.*, 1979). SDS-PAGE molecular weight standards were used as size markers (Sigma).

Northern Blot

Total RNA was extracted from the cells infected with the wild type AcNPV or BtrusI at 24 and 48 hr p.i. Cells were washed twice with PBS (pH 7.3) lacking calcium and magnesium ions. The cell pellets were suspended in 500 μl of the RNA extraction buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.6, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1,000 units/ml placental RNAase inhibitor). Then, the suspension was mixed with 500 μl of the proteinase digestion buffer (0.2 M Tris-HCl, pH 8.0, 25 mM EDTA, 0.3 M NaCl, 2% SDS), added proteinase K to a final concentration of 200 μg/ml, and then incubated at 37°C for 30 min. After incubation, total RNA was isolated with phenol/chloroform extraction. Northern blot analysis was carried out as described in Sambrook *et al.* (1989). Twenty micrograms of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde and blotted onto a membrane. The membrane was hybridized with the [^{32}P]-labeled AcNPV polyhedrin gene or the N-terminal region of the crystal protein gene.

Cysteine protease assay

Sf9 cells infected with the recombinant virus were cultured in TC-100 medium containing $2.8-5.6 \times 10^{-4}$ M E-64 cysteine protease inhibitor (Sigma) per ml medium (Ohkawa *et al.*, 1994). After incubation at 27°C for 48 hrs, total cellular lysates were harvested and subjected to SDS-PAGE and Western blot analysis.

Bioassay against *Bombyx mori* Larvae

Mock-infected Sf9 cells or Sf9 cells infected with recombinant AcNPV were inoculated into the second instar larvae of *B. mori*, which were then fed on an artificial diet at 25°C. Cells (1×10^7) infected with the wild type AcNPV or the recombinant virus were treated on the artificial diet. The insecticidal activity of the fusion protein produced by the recombinant virus was determined by mortality which was scored for 3 days after application of cell samples.

RESULTS AND DISCUSSION

Construction of Recombinant AcNPV

The baculovirus transfer vector to generate the recombinant virus expressing the fusion protein with AcNPV polyhedrin and Bt cryIA (c) crystal protein is described in Fig. 1. To confirm the introduction of the fusion gene, genomic DNA extracted from the recombinant viruses was analyzed by PCR (Fig. 1C) to show a 2.5 kbp band expected for the fusion gene.

Expression of the Fusion Gene in Insect Cells

Sf9 cells infected with BtrusI were produced polyhedra, which were similar , in appearence, to those from the wild type AcNPV (Fig. 2). However, the number of polyhedra produced by BtrusI was lower by approximately 100-fold compared with that of Sf9 cells inoculated with the wild type AcNPV. The average titer of recombinant baculovirus, BtrusI, was similar to that of wild type AcNPV (Table 1).

In order to examine expression of the fusion gene of recombinant virus in Sf9 cells, the protein synthesis in Sf9 cells infected with BtrusI was initially analyzed by SDS-PAGE and Western blot (Fig. 3). Wild-type AcNPV-infected cells showed a pattern of protein synthesis typical of AcNPV-infected cells. The fusion

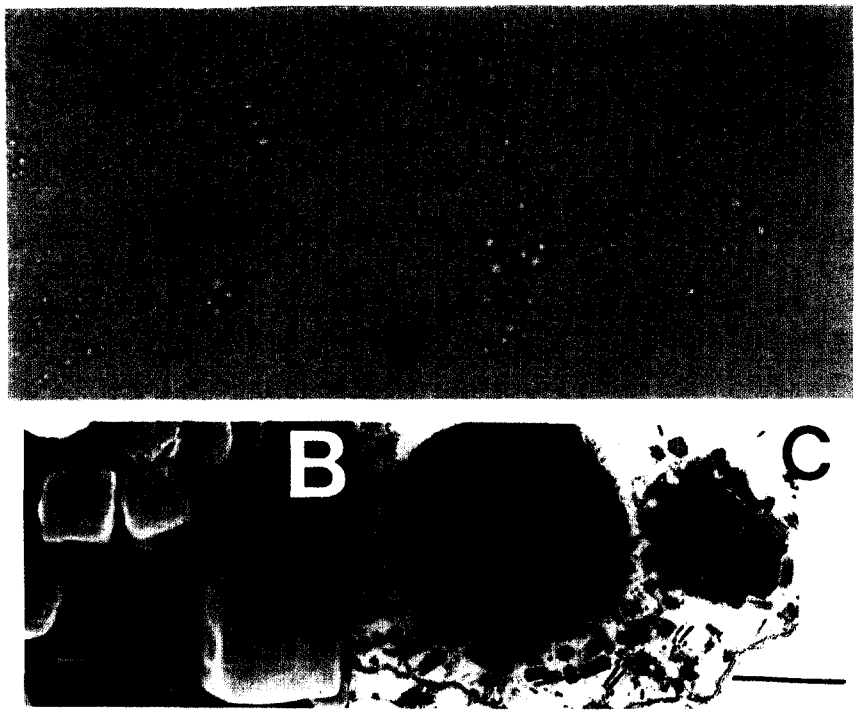

Fig. 2. Microscopy of polyhedra produced in Sf9 cells infected with recombinant virus BtrusI. A, Inverted phase contrast microscopy (×1,000); B, Scanning electron microscopy; C, transmission electron microscopy. Size bars indicate 1 μm.

Table 1. Titeration of the recombinant virus, BtrusI, in insect cells

Virus	Titer*
Wild-type AcNPV	6.2×10^7
BtrusI	5.6×10^7

* Virus titer was determined by the plaque assay.

protein was expressed as a band of about 92 kDa in cells infected with recombinant virus, BtrusI, but absent in cells infected with the wild type AcNPV. That the 92 kDa band is the fusion protein with the polyhedrin and the cryIA (c) crystal protein was demonstrated by immunoblotting with anti-polyhedrin or cryIA (c) crystal protein antibodies (Fig. 3B, C). However, the cells infected with BtrusI also showed a 33 kDa protein band as in cells infected with the wild type AcNPV. In addition, polyhedra purified from the cells infected with BtrusI showed a 33 kDa protein band only (Fig. 3A).

To determine the transcript of the fusion gene in BtrusI, total RNA was isolated from the cells infected with BtrusI and the transcripts were characterized by nothern blot analysis (Fig. 4). The result showed that the transcript of fusion gene was apparently present as a single band of about 2.5 kbp in cells infected with Btrus I, whereas the polyhedrin gene was revealed as a band of about 730 bp in cells infected with the wild type AcNPV. Although the transcript expected for the fusion protein gene was observed, the cells infected with BtrusI produced two major protein bands, fusion protein and polyhedrin. It has been reported that baculovirus genome includes cystein proteinase gene (Ohkawa *et al.*, 1994; Slack *et al.*, 1995). Therefore, our results suggested that fusion protein expressed by BtrusI was cleaved by the cystein proteinase of baculovirus and the cleaved polyhedrin was assembled with virus particles.

To test the possibility, we cultured Sf9 cells infected with BtrusI in the presence of proteinase inhibitor, E-64, and analyzed the production of the fusion protein

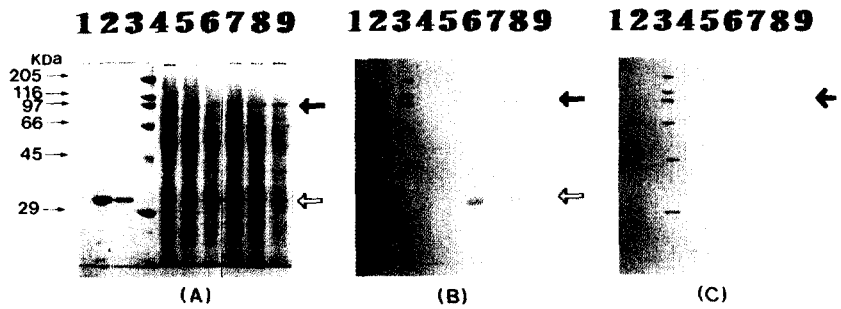

Fig. 3. SDS-PAGE and Western blot analysis of the fusion protein expression of recombinant virus, BtrusI, in Sf9 cells. Polyhedra were purified from the Sf9 cells infected with wild type AcNPV (lane 1) or BtrusI (lane 2). Sf9 cells were mock infected (lane 4) or infected with wild-type AcNPV (lanes 5 and 6) and BtrusI (lanes 7, 8, and 9) at MOI of 5 PFU per cell. Cells were collected at 1 (lanes 5 and 7), 2 (lanes 6 and 8) and 3 (lane 9) days p.i. Total cellular lysates were subjected to 10% SDS-PAGE (panel A), electroblotted and incubated with anti-polyherin (panel B) or crystal protein (panel C) antibody. Fusion protein (solid arrows) and polyhedrin (open arrows) bands are indicated on the right of each panel. Molecular weight standards were used as size markers (lane 3).

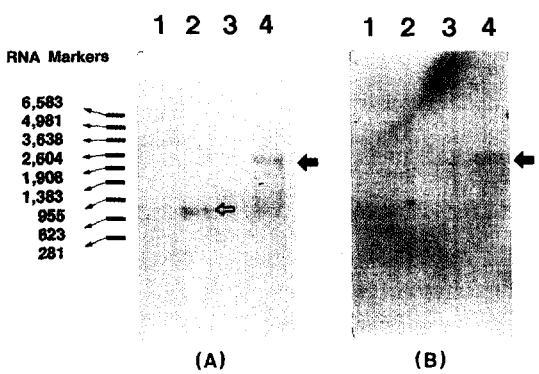

Fig. 4. Northern blot analysis of Sf9 cells infected with the recombinant virus BtrusI. Total RNA was extracted from the cells infected the wild type AcNPV (lanes 1 and 2) or BtrusI (lanes 3 and 4) at 24 (lanes 1 and 3) or 48 (lanes 2 and 4) hr p.i. Twenty micrograms of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde, and blotted onto a membrane. The membrane was hybridized with the [^{32}P]-labeled AcNPV polyhedrin gene (panel A) or N-terminal of crystal protein gene (panel B). Fusion gene (solid arrows) and polyhedrin gene (open arrow) transcripts are indicated on the right of each panel.

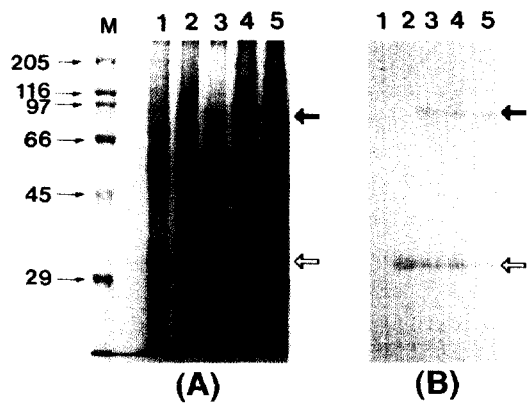

Fig. 5. SDS-PAGE and Western blot analysis of the fusion protein expression of a recombinant virus, BtrusI, in Sf9 cells treated with the cysteine protease inhibitor. Sf9 cells were mock infected (lane 1) or infected with wild-type AcNPV (lane 2) or BtrusI (lanes 3, 4, and 5) at MOI of 5 PFU per cell. Sf9 cells were cultured in TC-100 medium containing 2.8 (lane 4) - 5.6 (lane 5) $\times 10^{-4}$ M E-64 per ml medium. After incubation at 27°C, total cellular lysates were harvested from the cells at 48 hr p.i. Total cellular lysates were subjected to 10% SDS-PAGE (panel A), electroblotted and incubated with an anti-polyherin (panel B) antibody. Fusion protein (solid arrows) and polyhedrin (open arrows) bands are indicated on the right of each panel.

(Fig. 5). The result showed that expression level of the fusion protein in E-64 treated Sf9 cells was slightly lower than that of E-64 untreated Sf9 cells and the proteinase inhibitor, E-64, has no effect on the inhibition of clevage of the fusion protein.

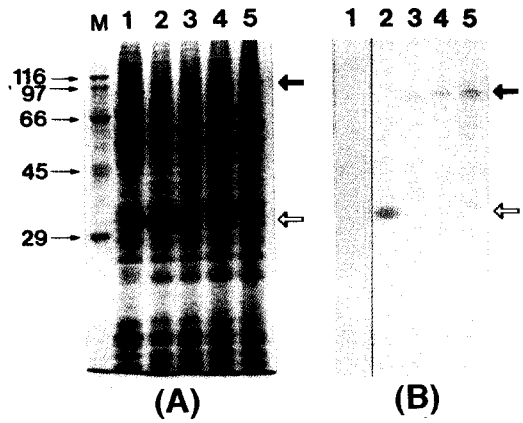

Fig. 6. SDS-PAGE and Western blot analysis of the fusion protein expression of a recombinant virus, BtrusII, in Sf9 cells. Sf9 cells were mock infected (lane 1) or infected with wild-type AcNPV (lane 2) or BtrusII (lanes3, 4, and 5) at MOI of 5 PFU per cell. Cells were collected at 1 (lane 3), 2 (lane 4) or 3 (lanes 2 and 5) days p.i. Total cellular lysates were subjected to 10% SDS-PAGE (panel A), electroblotted and incubated with an anti-polyhedrin (panel B) antibody. Fusion protein (solid arrows) and polyhedrin (open arrow) bands are indicated on the right of each panel.

For the purpose of stable expression and a new approach towards the production of fusion protein, BtrusII was constructed as described in Fig. 1B. The protein synthesis in Sf9 cells infected with BtrusII was also analyzed by SDS-PAGE and Western blot (Fig. 6). The fusion protein expressed by BtrusII was 92 kDa as in cells infected with BtrusI. That the 92 kDa band was the fusion protein with a polyhedrin and cryIA (c) crystal protein was demonstrated by immunoblotting. Interestingly, however, the cells infected with BtrusII did not show a 33 kDa polyhedrin band as in cells infected with BtrusI. Futhermore, cells infected with BtrusII did not produce polyhedra.

In this study, although a single transcript from the fusion protein gene was apparently detected, BtrusI produced two proteins, 92 kDa fusion protein and 33 kDa polyhedrin. These results indicate that the two proteins were produced from the fusion protein gene. This suggests that the proteins were produced by translation initiation at both fusion gene and polyhedrin gene initiator ATG codon (Beames *et al.*, 1991). In addition, the fusion protein produced by BtrusI did not form polyhedra. It is suggested that in the formation of inclusion body, substitution polyhedrin gene for strucural domain of C-terminal of Bt cryIA(c) crystal protein gene (Aronson *et al.*, 1986; Hofte & Whiteley, 1989) did not affect.

Bioassay against *B. mori* Larvae

Insecticidal activity of the fusion protein expressed by Sf9 cells infected with BtrusI was evaluated against second-instar larvae of *B. mori* and compared with Sf9 cells infected with AcNPV (Table 2). Mock-infected and wild type AcNPV served as negative controls. Larvae of *B. mori* were chosen for this assay because they are nonsusceptible to AcNPV but highly sensitive to the cryIA (c) crystal protein. Cells (1.0×10^7) infected with the wild type AcNPV or BtrusI were treated on the artificial diets, and the larval mortality was measured 3 days p.i. Sf9 cells infected with BtrusII that expressed the fusion protein caused larval mortality although the toxicity was low.

In conclusion, our results clearly demonstrated that the fusion protein with polyhedrin and Bt cryIA (c) crystal protein have an insecticidal activity. The fact that the fusion protein produced in baculovirus-infected insect cells is toxic provides the basis for a genetic engineering strategy in which Bt crystal protein genes are used to enhance baculovirus pathogenicity.

Table 2. Insecticidal activity of the fusion protein with a polyhedrin and *B. thuringiensis* cryIA(c) crystal protein produced by BtrusI-infected *S. frugiperda* cells against second-instar larvae of *B. mori*

Source	% Mortality*
S. frugiperda cells	0
S. frugiperda cells infected with wild type AcNPV	0
S. frugiperda cells infected with BtrusI	36.4

* Mortality was scored 3 days after application of cell samples.

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