Inactivation of *Bombyx mori* macula-like virus under physical conditions

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Abstract The *Bombyx mori* macula-like virus (BmMLV) is a member of the genus *Maculavirus*, family *Tymoviridae*, and contains a positive-sense single-stranded RNA genome. Previously, we reported that almost all *B. mori*-derived cell lines have already been contaminated with BmMLV via an unknown infection route. Since *B. mori*-derived cell lines are used for the baculovirus expression vector system, the invasion of BmMLV will cause a serious safety risk in the production of recombinant proteins. In this study, to determine the inactivation effectiveness of BmMLV, viruses were treated with various temperatures as well as gamma and ultraviolet (UV) light radiation. After these treatments, the virus solutions were inoculated into BmMLV-free BmVF cells. At 7 days postinoculation, the amount of virus in cells was evaluated by real-time reverse transcription PCR. Regarding heat treatment, conditions under 56°C for 3 h were tolerated, whereas infectivity disappeared after treatment at 75°C for 1 h. Regarding gamma radiation treatment, viruses were relatively stable at 1 kGy; however, their infectivity was entirely eliminated at a dose of 10 kGy. With 254 nm UV-C treatment, viruses were still active at less than 120 mJ/cm^2; however, their infectivity was completely lost at greater than 140 mJ/cm^2 UV-C radiation. These results provide quantitative evidence of the potential for BmMLV inactivation under a variety of physical conditions.

Keywords Maculavirus · BmMLV · BEVS · Heat inactivation · Gamma radiation · UV-C radiation

Introduction

The baculovirus expression vector system (BEVS) is a powerful eukaryotic method for producing difficult-to-express recombinant proteins with high yields. Since many of the post-translational modifications present in eukaryotes are performed in baculovirus-infected insect cells, the overexpressed protein exhibits the proper biological activity and function. Accordingly, the BEVS has been utilized to develop next-generation vaccines, vectors for gene therapy, and other biopharmaceutical complex proteins (reviewed in Felberbaum 2015). Two of the most common baculovirus species used in the BEVS are *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and *Bombyx mori* NPV (BmNPV) belonging to the group I *Alphabaculovirus* (reviewed in Rohrmann 2013). Mammalian cytokines have been produced using recombinant BmNPVs and were launched as antiviral and atopic dermatitis drugs for companion animals (reviewed in Kato *et al.* 2010). Recently, an influenza virus-like particle was produced in silkworm pupae infected with recombinant BmNPV (Nerome *et al.* 2015). For the BmNPV-based BEVS, *B. mori*-derived cell lines that are susceptible to BmNPV are indispensable for the generation

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and/or propagation of recombinant BmNPVs and production of recombinant proteins.

Previously, we reported that a *B. mori* ovary-derived cell line, BmN4, was persistently infected with a *B. mori* macula-like virus (BmMLV) (Katsuma *et al.* 2005). BmMLV is a member of the genus *Maculavirus*, family *Tymoviridae*, and contains a positive-sense single-stranded RNA genome. Interestingly, almost all *B. mori*-derived cell lines have already been contaminated with BmMLV via an unknown infection route (Iwanaga *et al.* 2012). As described above, since *B. mori*-derived cell lines are frequently used for BmNPV-based BEVS for the production of veterinary medicines, the contamination of *B. mori* cell lines with BmMLV will be a higher safety risk in the production of recombinant proteins.

To inactivate the virus, treatments with heat as well as gamma and ultraviolet (UV) light radiation are frequently used (Smith 1962; Spire *et al.* 1985; Burnouf and Radosevich 2000; Lowy *et al.* 2001; Thurston-Enriquez *et al.* 2003; De Benedictis *et al.* 2007; Song *et al.* 2010; Nims *et al.* 2011; Zou *et al.* 2013). This study evaluated the efficacy of several viral inactivation methods in eliminating BmMLV infectivity, including heat treatment and gamma and UV-C light radiation. Virus infectivity was assessed by real-time reverse transcription PCR (RT-PCR) and Western blot analysis. The results revealed that all of these methods were able to inactivate BmMLV.

Materials and Methods

Cells, the virus, and viral inoculation. *B. mori*-derived BmMLV-positive BmN4 (Grace 1967) and BmMLV-negative BmVF (Iwanaga *et al.* 2012) cell lines were maintained as described previously on TC-100 and IPL-41 media, respectively, supplemented with 10% fetal bovine serum (FBS) (Iwanaga *et al.* 2004, 2012). BmMLV solution was prepared from BmN4 cells as described previously (Iwanaga *et al.* 2012). After viral inactivation treatments, 2×10^6 BmVF cells were inoculated with 4×10^4 BmN4 cell-derived BmMLV solution and incubated for 1 h. After virus inoculation, cells were washed twice with phosphate-buffered saline (PBS) and transported to a 60-mm culture dish. Samples were collected with a cell scraper at 7 days postinnoculation (dpi) and subjected to real-time RT-PCR and Western blot analysis.

Real-time RT-PCR analysis. Total RNAs were prepared from BmMLV-inoculated BmVF cells by using RNAiso Plus reagents (Takara Bio, Shiga, Japan), and then, the first-strand complementary DNA (cDNA) was synthesized from 500 ng of total RNA with random hexamers and the PrimeScript RT reagent kit (Takara Bio). Subsequently, cDNA fragments were subjected to real-time PCR analysis using primers q-BmMLV-cp-F (5′-TTGACCTTTGTTGGACTACTGCTG-3′) and q-

BmMLV-cp-F (5′-GATGACATCGACGATGATCCAAATG-3′) for BmMLV *coat protein* (*cp*) and qRT-actin-F (5′-GATGACATCGACGATGATCCAAATG-3′) and qRT-actin-R (5′CTCGGTCCTGTCTGAGCTCTCTTG-3′) for *B. mori actin*, which was performed by using the SYBR Premix Ex Taq II (Takara Bio) and the ABI PRISM 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA). Statistical analysis was carried out using one-way ANOVA and Tukey post hoc test with KaleidaGraph computer software version 4.1 (Synergy Software, Reading, PA).

Western blot analysis. Cell extracts were prepared from BmMLV-inoculated BmVF cells, electrophoresed, transferred on a Hybond ECL membrane (GE Healthcare, Buckinghamshire, UK), and subjected to Western blotting. Antibodies of BmMLV CP (Iwanaga *et al.* 2012) and beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used as probes. Anti-rabbit IgG conjugated to horseradish peroxidase (KPL, Gaitherburg, MD) and the Western blot substrate plus (Thermo Fisher Scientific, Waltham, MA) were used. Signal detection was carried out using the Light Capture II system (ATTO, Tokyo, Japan).

Heat treatment. The viral solution was dispensed into 100 μl aliquots in tightly capped, 1.5-ml microcentrifuge tubes and then incubated in a dry bath with temperatures of 25, 37, 45, 56, 65, 75, and 100°C. At designated time points, samples were removed and transferred immediately into an ice-water bath to stop the effect of the heat. Subsequently, heat-denatured proteins were removed by centrifuging at 12,000×*g* at 4°C for 10 min, and then, the supernatants were used for virus inoculation.

Gamma radiation. The viral solution was dispensed into 100 μl aliquots in tightly capped, 1.5-ml cryotubes and then exposed to doses of 0, 0.05, 0.1, 0.2, 0.5, 1, 10, 20, and 40 kGy of gamma radiation from a ^{60}Co source using a gammacell (model PIIC-480C; Pony Industry, Osaka, Japan) with an average dose rate of approximately 141 Gy/min during the radiation exposure. The samples were kept at 4°C by adding crushed ice during gamma irradiation. After irradiation, samples were used as a virus solution.

UV-C light radiation. The viral solution was placed on a parafilm (Bemis Company, Oshkosh, WI) and then exposed to a continuous 254-nm UV-C light at 0, 20, 40, 60, 100, 120, 140, 160, and 180 mJ/cm^2, using a CL-1000 Ultraviolet Crosslinker (UVP, Upland, CA). Experiments were performed at room temperature. After radiation, samples were sterilized using a 0.2-μm syringe filter and then used for virus inoculation.

Results

Effect of heat treatment on BmMLV infectivity. To evaluate the sensitivity of BmMLV to heat treatment, aliquots of BmMLV solution (derived from 4×10^4 BmN) were treated at seven temperatures (25, 37, 45, 56, 65, 75, and 100°C) for 30 min. As shown in Fig. 1*A*, with treatment at 75°C, the viral RNA at 7 dpi significantly decreased to 3.4% of that at 25°C ($p<0.001$). Also, the RNA amounts sharply reduced to less than 0.1% with treatment at 100°C. To assess the effect of the incubation length on virus infectivity, viral solutions were treated at 56 and 75°C for 1, 2, and 3 h. As shown in Fig. 1*B*, while there were no significant differences when treated at 56°C for 2 h, the viral RNA significantly decreased

to 25.1% with treatment for 3 h ($p<0.005$). Also, the viral RNA completely diminished with treatment of more than 1 h at 75°C ($p<0.001$). These results indicate that BmMLV can be completely inactivated with treatment at 75°C for more than 1 h or 100°C for 30 min.

Effect of gamma-ray radiation on BmMLV infectivity. Next, to evaluate the effect of gamma radiation on BmMLV infectivity, aliquots of BmMLV solution (derived from 4×10^4 BmN) were given doses of 0 to 40 kGy from a ^{60}Co source of gammacell. As shown in Fig. 2, although viral RNA at 7 dpi declined to 50.2% with 1 kGy radiation, there was no significant difference between 0 and 1 kGy radiation ($p>0.001$). However, the viral RNA abruptly disappeared following treatment with more than 10 kGy radiation ($p<0.001$) (Fig. 2), thereby indicating that BmMLV can be completely inactivated by more than 10 kGy of gamma-ray radiation.

Effect of UV-C light radiation on BmMLV infectivity. Finally, to examine the effect of continuous UV-C light on BmMLV infectivity, aliquots of BmMLV solution (derived from 4×10^4 BmN) were placed on a parafilm and subjected to UV-C light radiation from 0 to 180 mJ/cm². As shown in Fig. 3, although the viral RNA at 7 dpi declined to 34.9% with treatment of 100 mJ/cm², there were no significant differences between 0 and 100 mJ/cm² ($p>0.001$). On the other hand, the RNA amounts were sharply reduced to less than 1% following treatment with 120 mJ/cm² radiation ($p<0.001$). Also, the viral RNA was completely diminished with treatment of more than 140 mJ/cm² UV-C light ($p<0.001$). These results indicate that BmMLV can be completely inactivated by more than 140 mJ/cm² UV-C radiation.
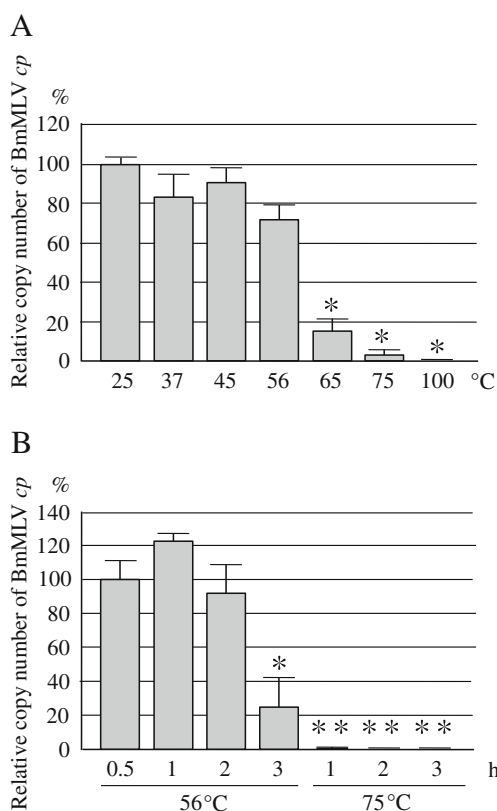

Figure 1 Inactivation of BmMLV by heat treatment. (*A*) The viral solution was incubated at temperatures of 25, 37, 45, 56, 65, 75, and 100°C for 30 min and then inoculated into BmMLV-negative BmVF cells. At 7 days postinoculation (dpi), total RNA was prepared from the cells, reverse transcribed, and subjected to real-time PCR analysis of the *cp* gene. The results represent the average of three independent experiments. *Asterisk* indicates a result significantly different from that obtained with treatment at 25°C (one-way ANOVA, *$p<0.001$). Standard errors are indicated. (*B*) The viral solution was incubated at temperatures of 56 or 75°C for designated time points and then inoculated into BmMLV-negative BmVF cells. At 7 dpi, total RNA was prepared from the cells, reverse transcribed, and subjected to real-time PCR analysis of the *cp* gene. The results represent the average of three independent experiments. *Asterisk* indicates a result significantly different from that obtained with treatment at 56°C for 30 min (one-way ANOVA, *$p<0.005$, **$p<0.001$). Standard errors are indicated.
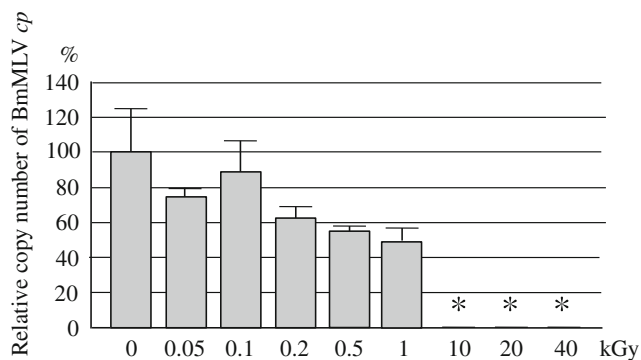

Figure 2 Inactivation of BmMLV by gamma radiation. The viral solution was exposed to gamma rays at doses of 0, 0.05, 0.1, 0.2, 0.5, 1, 10, 20, and 40 kGy from a ^{60}Co source and was then inoculated into BmMLV-negative BmVF cells. At 7 dpi, total RNA was prepared from the cells, reverse transcribed, and subjected to real-time PCR analysis of the *cp* gene. The results represent the average of three independent experiments. *Asterisk* indicates a result significantly different from that obtained with 0 kGy radiation (one-way ANOVA, *$p<0.001$). Standard errors are indicated.
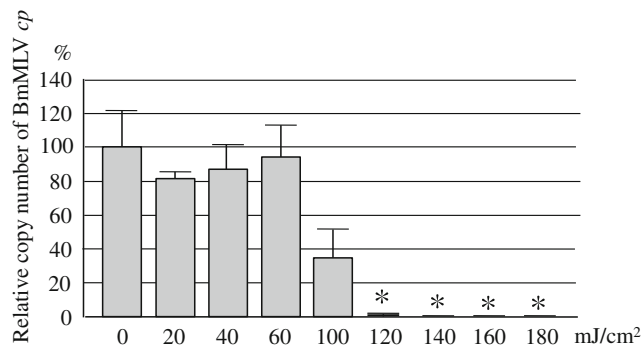
Figure 3 Inactivation of BmMLV by UV-C radiation. The viral solution was exposed to continuous UV-C light at 0, 20, 40, 60, 100, 120, 140, 160, and 180 mJ/cm^2 and then inoculated into BmMLV-negative BmVF cells. At 7 dpi, total RNA was prepared from the cells, reverse transcribed, and subjected to real-time PCR analysis of the *cp* gene. The results represent the average of three independent experiments. *Asterisk* indicates a result significantly different from that obtained with 0 mJ/cm^2 radiation (one-way ANOVA, *$p<0.001$). Standard errors are indicated.

Confirmation of BmMLV inactivation by Western blot analysis. To verify the results of real-time RT-PCR analysis, 1×10^5 BmVF cells were inoculated with BmMLV solution that was inactivated by heat, gamma ray, or UV-C. At 7 dpi, cell extracts were electrophoresed and subjected to Western blotting with anti-CP antibody. As shown in Fig. 4, while a viral CP signal was detected with treatment at 56°C for 3 h, no signal was detected with treatment at 75°C for 1, 2, or 3 h. Also, distinct viral CP signals were detected when treated with both 0.5 and 1 kGy gamma-ray radiation; however, there was no detectable signal when treated with 10 kGy radiation (Fig. 4). Furthermore, although there were clear viral CP signals during treatment with both 100 and 120 mJ/cm^2 UV-C radiation, no detectable signal was observed during treatment with 140 mJ/cm^2 radiation (Fig. 4). Combined with the results of real-time RT-PCR analysis (Figs. 1, 2, and 3), we concluded that BmMLV was inactivated with treatment at 75°C for 1 h, 10 kGy gamma radiation, and 140 mJ/cm^2 UV-C radiation.
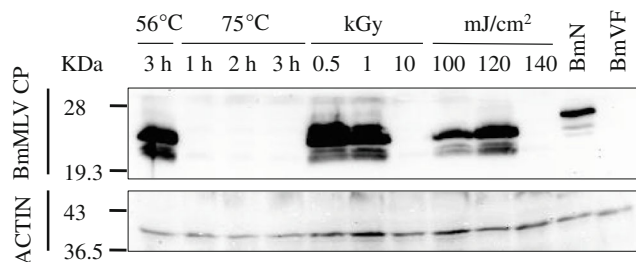

Figure 4 Inactivation of BmMLV under physical conditions. The viral solution was treated with heat and gamma and UV-C radiation before being inoculated into BmMLV-negative BmVF cells. At 7 dpi, extracts were prepared from 1×10^5 viral solution-inoculated cells and were subjected to SDS-PAGE, followed by Western blotting with anti-BmMLV CP and actin. Size markers are indicated on the *left side* of each *panel*. BmMLV-positive BmN4 and BmMLV-negative BmVF cells were used as positive and negative controls, respectively.

Discussion

In this study, a detailed analysis was conducted as to the efficacy of several conventional viral inactivation procedures, i.e., heat treatment and gamma and UV-C radiation for eliminating BmMLV infectivity.

Heat treatment is a widely used viral inactivation method that is effective against both enveloped and nonenveloped viruses (Burnouf and Radosevich 2000). Since high temperature causes the denaturation of viral proteins, some enveloped viruses are easily inactivated by heat treatment. The infectivity of both lymphadenopathy-associated virus, of the family *Retroviridae*, and avian influenza A (H7N9), of the family *Orthomyxoviridae*, is entirely eliminated with treatment at 56°C for 30 min (Spire *et al.* 1985; Zou *et al.* 2013). On the other hand, the family *Tymoviridae* generally survives at slightly higher temperatures, and the thermal inactivation point is 60–65°C to greater than 80°C for some tymoviruses (Martelli *et al.* 2002). In the current study, BmMLV showed heat tolerance at both 56°C for 3 h and 75°C for 30 min (Figs. 1 and 4). Considering that BmMLV belongs to the family *Tymoviridae*, the virion structure of this family may be resistant to heat treatment. As shown in Fig. 4, inactivation of BmMLV succeeded with treatment at 75°C for 1 h. However, this condition may cause serious denaturation of the recombinant protein expressed by BEVS. The heat inactivation method may be applied to the expression of specified low molecular weight peptides.

Gamma-ray treatment is a powerful tool for viral inactivation. A total radiation dose of 25–40 kGy fully inactivates many viruses and mycoplasma in animal sera (reviewed in Nims *et al.* 2011). In the current study, real-time RT-PCR and Western blot analysis showed that the infectivity of BmMLV can be entirely eliminated by gamma radiation at a dose of 10 kGy (Figs. 2 and 4). For BEVS, it is important to prevent the denaturation of recombinant proteins. In general, gamma radiation inactivates microorganisms through direct and indirect actions (Thomas *et al.* 1981). Direct action is a result of interactions of the radiation with nucleic acids of the microorganism that can cause base mutations, strand cross-linking, and strand breakage. Indirect action consists of the radiation's interaction with the milieu and includes the creation of free radicals (Sommer *et al.* 2001). Therefore, in the case of the influenza virus, the primary targets by which gamma radiation brings about virus inactivation are strand breaks of viral nucleic acids rather than denaturation of the virion structure (reviewed in De Benedictis *et al.* 2007). Also, gamma radiation inactivates viruses without detrimentally impacting the antigenic structure and biological integrity of proteins (Lowy *et al.* 2001). Therefore, the gamma radiation method for inactivating BmMLV represents a major advantage in the expression of recombinant proteins using BEVS.

UV-C radiation is another commonly used physical method for viral inactivation. This method inhibits viral replication by inducing the formation of thymine dimmers, a pyrimidine base (Perdiz *et al.* 2000). It was reported that the doses of UV required to achieve 99% inactivation of adenovirus type 40, of the family *Adenoviridae*, and feline calicivirus, of the family *Caliciviridae*, were 109 and 16 mJ/cm^2, respectively (Thurston-Enriquez *et al.* 2003). Additionally, the hepatitis C virus, of the family *Flaviviridae*, was inactivated completely by UV-C radiation at a dose of 27 mJ/cm^2 (Song *et al.* 2010). In the current study, the infectivity of BmMLV was fully inactivated by 140 mJ/cm^2 UV-C radiation (Figs. 3 and 4). Since UV-C light causes a protein-nucleic acid cross-link that plays a significant role in the inactivation of the microorganism (Smith 1962), it will be necessary for BEVS to confirm whether the recombinant protein exhibits the proper biological activity and functions under this UV-C-mediated viral inactivation method. Additionally, it is well known that gamma radiation is the most expensive method, and thus, UV-C light methods may prove to be more suitable for small-scale BEVS.

In the present study, several physical treatments for eliminating BmMLV were evaluated. There is a possibility that these virus inactivation methods can be applied to at least three steps. The first step is to inactivate BmMLV in the recombinant protein-containing solution after biochemical purification because we cannot exclude the possibility that BmMLV is still contaminated. Some proteins are sensitive to heat treatment; thus, application of heat inactivation methods depends on the feature of the recombinant protein expressed by BEVS. Also, since the target of gamma and UV-C light radiation is the nucleic acid of viral genome, these inactivation methods may be useful to inactivate BmMLV in the recombinant protein-containing solution. The second step is to inactivate BmMLV in the recombinant BmNPV-containing solution. Since BmNPV could be damaged by gamma and UV-C radiation, it may be difficult to selectively inactivate BmMLV in the recombinant BmNPV-containing solution by these methods. The third step is to inactivate BmMLV when researchers maintain BmN4 cells. However, it is somewhat difficult to maintain BmN4 cells under BmMLV-free condition because the origin of this virus is still unknown. In this case, it is better to use BmMLV-free cell line VF that we have established before (Iwanaga *et al.* 2012, 2014). In conclusion, this study demonstrated that all physical methods we tested can completely inactivate BmMLV, indicating that these inactivation methods could be applied to BmNPV-based BEVS.

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