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NOTE

Evaluation of viral contamination in a baculovirus expression system

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ABSTRACT

Insect expression systems based on baculovirus are widely used for generating recombinant proteins. Here, the infectivity of baculoviruses under the physiological stresses of 'freeze–thaw' and sonication and the baculoviral contamination of recombinant proteins after protein purification were evaluated. Our findings suggest that Nonidet P-40 (NP-40) treatment of baculoviruses completely abolishes their infectivity and that recombinant proteins purified with affinity beads do not include infectious baculoviruses. We therefore suggest that baculovirus is completely inactivated by NP-40 treatment and that recombinant proteins are unlikely to be contaminated with infectious baculoviruses after their affinity purification.

Key words baculovirus, insect expression system.

Baculovirus expression systems are powerful tools for generating recombinant proteins (1–3). The Cartagena Protocol on Biosafety to the Convention on Biological Diversity is an international agreement to ensure that adequate safety is maintained when living gene-modified organisms are handled or transported. Therefore, generation of recombinant baculoviruses and use of the commercially available recombinant proteins produced by baculoviruses must be regulated in accordance with the terms of the protocol by the signatory countries (4). However, it is unclear whether the purified fractions of recombinant proteins produced with a baculovirus system contain infectious viral particles. In this study, we examined various effects of the protein purification steps on the infectivity of baculovirus.

First a recombinant baculovirus expressing secreted Nluc, designated rBV-Nluc, was generated (Fig. 1a) using a Bac-to-Bac Baculovirus Expression System (Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturer's protocol (2, 3, 5). PFU were determined with a standard plaque assay (6). Sf-9 cells (1×10^5) were inoculated with various infectious titers of rBV-Nluc and incubated for 96 hr in Sf-900 II SFM medium (Thermo Fisher Scientific) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA). Nluc activity in the culture supernatants of cells inoculated with 0.01–10,000 PFU/10^5 cells (MOI 10^{-7}–10^{-1}) with the recombinant virus was evaluated using the Nano-Glo Luciferase Assay System (Promega, Madison, WI, USA) (Fig. 1b). Nluc activity was detected in cells infected with 0.1 PFU of baculovirus. A recent study showed that plaque formation occasionally requires more than one infectious virus to achieve efficient infection (7), because even when a virus infects its target cells, viral propagation may be insufficient to form clear plaques. Our findings suggest that evaluation of Nluc activity is more sensitive than a conventional plaque assay for detecting an infectious virus.

Next, because both these techniques are commonly used in protein purification, the effects of physiological stresses, such as freeze–thaw and sonication, on

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List of Abbreviations: Nluc, NanoLuc luciferase; NP-40, Nonidet P-40; PFU, plaque-forming units; rBV, recombinant baculovirus; rBV-Nluc, recombinant baculovirus expressing secreted Nluc; Sf-9, *Spodoptera frugiperda*.

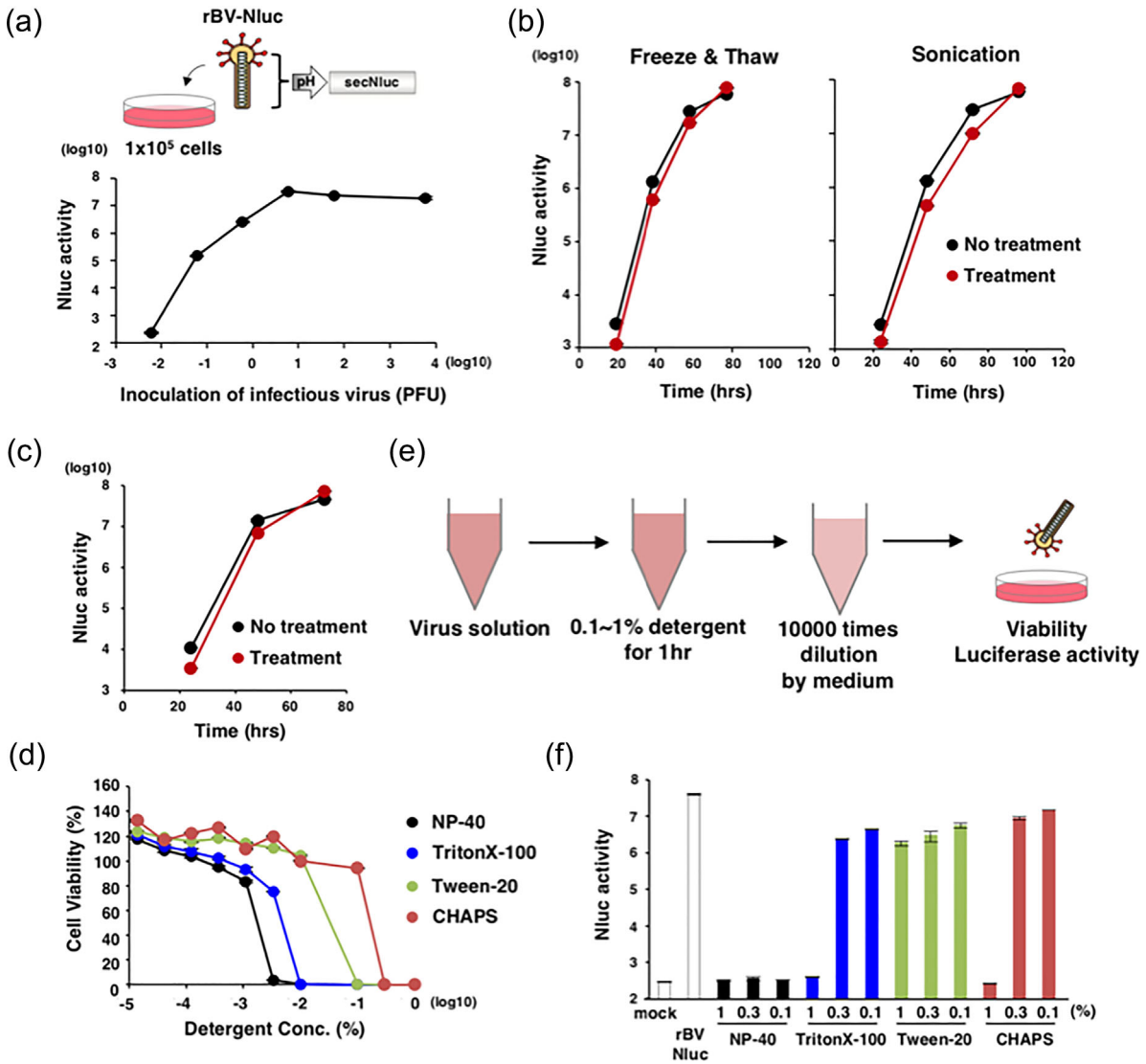

Fig. 1. Physiological features of the baculovirus. (a) Recombinant virus rBV-Nluc was generated with the Bac-to-Bac Baculovirus Expression System, used to inoculate naïve Sf-9 cells (in 24-well plates; Greiner) at indicated titers to 10^5, and incubated for 96 hr. Nluc activity in 10 μL of culture supernatant was measured. (b) Culture supernatants including 6×10^5 PFU of rBV-Nluc were treated with freeze–thaw (left panel) or sonication (right panel) and naïve Sf-9 cells (10^5) inoculated with the resulting solutions. Culture supernatants were harvested at the indicated times and subjected to Nluc assay. (c) Culture supernatants including 6×10^5 PFU of rBV-Nluc were centrifuged at 10,000 g for 10 min and then used to inoculate 10^5 naïve Sf-9 cells. The supernatants were subjected to Nluc assay. (d) NP-40, Triton X-100, Tween 20 or CHAPS was diluted to the indicated final concentrations in Sf-900 II SFM and added to culture supernatants of Sf-9 cells. After 1 hr, the culture mediums were replaced with SF-900 II SFM lacking detergent. The cells were incubated for a further 24 hr and then subjected to a cell viability assay with a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega). (e) Viral solutions were added at a final concentration of 3×10^6 PFU/mL to the indicated concentration of each detergent, incubated at 4°C for 1 hr, and then diluted 10,000 times with fresh medium (3×10^2 PFU/mL). Naïve Sf-9 cells (10^5) were inoculated with 0.2 mL of the diluted solutions and incubated at room temperature for 1 hr. After the mediums had been replaced with detergent-free medium, the cells were incubated for a further 96 hr and Nluc activity in the culture supernatants determined. (f) Virus solution (3×10^6 PFU/mL) was treated with the indicated concentrations of each detergent at 4°C for 1 hr and then diluted 10,000 times with fresh medium. Sf-9 cells (10^5) were inoculated with 0.2 mL of the resulting solution. The culture supernatants were harvested after 97 hr and subjected to a luciferase assay. Conc., concentration.

rBV-Nluc infectivity were tested. rBV-Nluc was frozen at −80°C and then thawed at room temperature in a water bath or treated with ultrasound (Bioruptor; Diagenode, Denville, NJ, USa) for 10 min. Both treatments resulted in a slight reduction in viral infectivity (Fig. 1b). Next, the effects of centrifugation (10,000 g, 10 min) on viral

infectivity were examined. Nluc activity was slightly reduced by centrifugation (Fig. 1c). These data suggest that freeze–thaw, sonication and centrifugation reduce infectivity, but do not completely inactivate the baculovirus. The effects of detergent treatment on viral infectivity were then assessed. First, the cytotoxicity of various detergents on Sf-9 cells was tested. The detergents Nonidet P-40 (NP-40, #25223; Nakalai Tesque, Kyoto, Japan), Triton X-100 (#35501; Nakalai Tesque), Tween 20 (#35624; Nakalai Tesque), and CHAPS (#07957; Nakalai Tesque) showed cytotoxicity of 0.001%–1% (Fig. 1d). A viral solution (3×10^6 PFU/mL) was treated with 0.1%, 0.3% or 1% detergent at 4°C for 1 hr, and then diluted 10,000-fold (3×10^2 PFU/mL). Sf-9 cells (1×10^5) in a 24 well plate were inoculated with 0.2 mL of the diluted solutions and incubated for 96 hr (Fig. 1e). Nluc activity was determined and the results indicated that treatment with NP-40, but not the other detergents, completely and efficiently abolished the infectivity of the baculovirus (Fig. 1f). These data suggest that baculoviruses have differing sensitivities to detergents and that, of the detergents tested in this study, baculovirus is the most sensitive to NP-40. It is unclear why NP-40 so effectively abolishes the infectivity of baculovirus; however, it has also been shown that NP-40 inactivates human T-cell lymphotropic virus type III/lymphadenopathy-associated virus (8).

Next, whether the recombinant baculovirus nonspecifically binds Sepharose beads during affinity purification was examined. Sf-9 cells were infected with rBV-Nluc and incubated for 7 hr. The culture was centrifuged at 10,000 g for 10 min and the supernatant collected. A portion of the supernatant was designated Sample A, added to 500 μL of Strep-Tactin Sepharose beads (IBA GmbH, Göttingen, Germany) (50% [v/v] in PBS) and incubated overnight at 4°C. The beads were washed three times with buffer A and eluted with 500 μL of elution buffer (buffer A containing 2.5 mM d-desthiobiotin) (9). The eluted sample was designated Sample B. Because rBV-Nluc does not express Strep-tag II peptide, Sample B was unlikely to contain either viral-derived proteins or infectious virus.

To determine whether Sample B was contaminated with infectious virus, 1×10^5 Sf-9 cells were inoculated with 100 μL of either Sample A or B. The culture supernatant was harvested 96 hr after infection and its NLuc activity determined. Representative data from two independent experiments are shown in Figure 2.

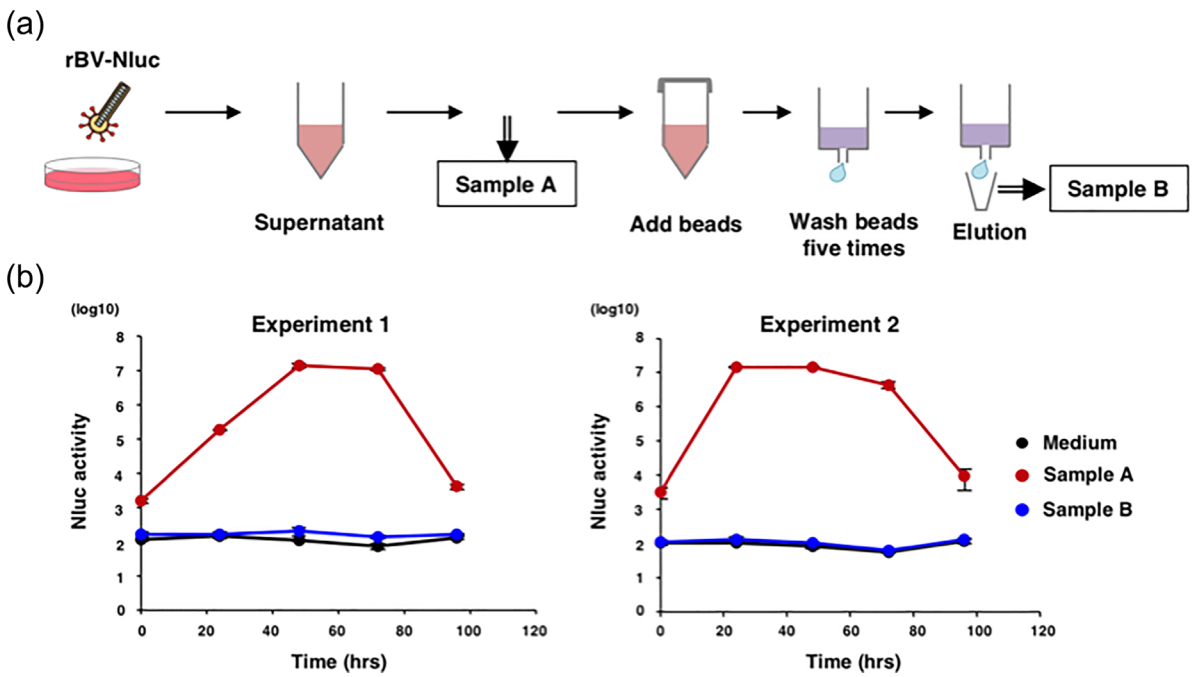

Fig. 2. Effects of affinity purification on contaminating infectious baculovirus. (a) Sf-9 cells were inoculated with rBV-Nluc at MOI 0.3 and incubated for 72 hr. The culture supernatants (0.5 mL) were collected and centrifuged at 1500 × g for 5 min (Sample A). The supernatants (50 mL) were mixed with 0.5 mL of 50% (v/v) Strep-Tactin beads and incubated at 4°C overnight. The beads were washed three times with buffer A and eluted with buffer A containing 2.5 mM d-desthiobiotin. The eluent was used as Sample B. (b) Naïve Sf-9 cells (10^5) were inoculated with each sample (0.1 mL) and incubated at room temperature for 1 hr. The culture medium was then replaced with fresh medium and the cells incubated at 26°C for 96 hr. Nluc activity in the culture supernatant was measured and is shown as the mean ± SD of two independent experiments.

Table 1. Recombinant proteins used in this study

No	Recombinant proteins	Manufacturer	Forms	Volume used for infection/total volume (μL)	Purification method
1	Human MRP2	GenoMembrane (Yokohama, Japan)	Tri-HCl solution	100/500	Plasma membrane fraction
2	Human CYP3A4 +Oxidoreductase+cytochrome b5	Corning (Corning, NY, USA)	Na3PO4 solution	100/500	Microsome fraction
3	Mouse CXCL1	R&D Systems (Minneapolis, MN, USA)	Lyophilized	100/500	Affinity
4	Human IL-10	R&D Systems	Lyophilized	100/500	Affinity
5	Human IL-10	Sigma (St. Louis, MO, USA)	Lyophilized	100/500	Affinity
6	Human IL-10	Perkin Elmer (Waltham, MA, USA)	Lyophilized	100/500	Affinity
7	Macaca IL-10	MyBioSource (San Diego, CA, USA)	Tri-HCl solution	100/500 or 10/500	Affinity

As expected, significant Nluc activity was detected in Sample A, but not in Sample B (Fig. 2b). These data suggest that the infectious virus does not bind nonspecifically to Sepharose beads and can be washed from them in the flow-through. Therefore, we suggest that an affinity-purification step based on Sepharose beads removes contaminating infectious viral particles.

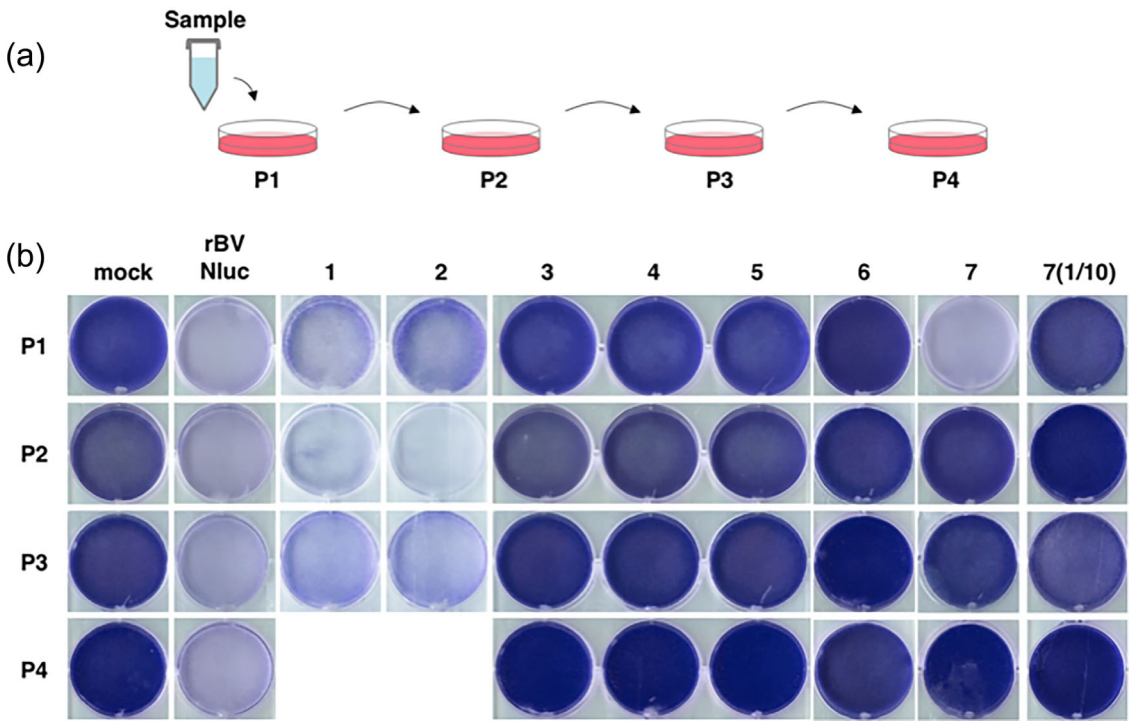

Fig. 3. Possible contamination of commercially available recombinant proteins with infectious virus. (a) Sf-9 cells were inoculated with 100 μL of recombinant protein and 400 μL of Sf-900 II SFM (total 500 μL) for 1 hr. The culture medium was then replaced with fresh medium and the cells cultured for a further 96 hr (P1). Fresh Sf-9 cells were inoculated with the culture supernatants for 1 hr, the medium replaced with fresh culture medium and the cells cultured for 96 hr (P2). This blind passage was continued to P4. (b) At each blind passage, the cells attached to the culture dishes were fixed with 4% paraformaldehyde and stained with crystal violet to estimate cytotoxicity. Because Sample 7 was toxic to Sf-9 cells at P1, but not at P2, Sf-9 cells were inoculated with a 10-fold dilution of the sample, shown as 7(1/10) and blind passages performed. The data are representative of three independent experiments.

Finally, whether commercial recombinant proteins produced in baculovirus systems contain infectious viral particles was examined. Although the details of the purification steps used for all the recombinant proteins listed in Table 1 are not available, the methods of protein purification can be divided into two techniques, subcellular fractionation and affinity purification. Sf-9 cells were treated with 0.1 mL of each protein solution and incubated at room temperature for 1 hr. The cells were then washed three times with fresh medium and incubated further at 26°C for 96 hr. The culture supernatant was designated P1. Naïve Sf-9 cells were treated with 0.1 mL of P1 as described above. The resulting supernatant was designated P2. The same cell culture procedure was repeated twice more, as shown in Figure 3a. The supernatants harvested from the third and the fourth passages were designated P3 and P4, respectively. The attached cells at each passage were fixed with 4% paraformaldehyde and stained with crystal violet (Fig. 3b) to visualize the remaining cells.

The protein aliquots prepared with subcellular fractionation from the P1, P2 and P3 preparations showed cytotoxicity, indicating that infectious viral particles were present in the recombinant protein preparations isolated with the subcellular fractionation technique (Samples 1 and 2; Fig. 3b). The only protein aliquot prepared with affinity purification that showed cytotoxicity was Sample 7 from P1; all other protein aliquots showed no cytotoxicity. These data suggest that the cytotoxic effect of P1 may be attributable to an ingredient(s) other than an infectious viral particle. Treatment of the cells with 10-fold-diluted Sample 7 showed no cytotoxicity against P1, P2, P3 or P4 (right end of Row) (Fig. 3b). These data suggest that no infectious particles were present in the recombinant proteins prepared with affinity purification.

The data reported in this study suggest that baculovirus infectivity is completely abolished by treatment with NP-40 and that affinity purification removes contaminating infectious viral particles from recombinant protein preparations. Our data also suggest that no infectious viral particles are present in commercially available samples prepared with affinity purification. Thus, NP-40 treatment and subsequent affinity purification can completely prevent contamination of recombinant protein preparations with infectious viral particles. The newly designed recombinant baculovirus that expresses Nano-Luc luciferase (rBV-Nluc) is useful for the highly sensitive detection of infectious viral particles.

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DISCLOSURE

The authors declare that they have no conflicting interests.

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