Appl Microbiol Biotechnol (2008) 78:221–226
DOI 10.1007/s00253-007-1296-y

Efficient production of canine interferon-alpha in silkworm *Bombyx mori* by use of a BmNPV/Bac-to-Bac expression system

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Received: 29 September 2007 / Revised: 20 November 2007 / Accepted: 21 November 2007 / Published online: 8 December 2007

Abstract We exploited the silkworm *Bombyx mori* for the production of recombinant canine interferon-alpha (CaIFN-α). The recombinant baculovirus harboring canine interferon gene was rapidly generated by the BmNPV/Bac-to-Bac system that was recently developed. In *B. mori*-derived cell lines, the expression of the recombinant protein reached maximal levels around 72–96 h post-infection. For the isolation of the expressed recombinant protein from *B. mori* larvae, the whole bodies of the infected larvae were homogenized, and the expressed protein was purified by affinity chromatography. Based on the fact that the recombinant CaIFN-α showed two bands on the sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern, the expressed protein was thought to be glycosylated. The rCaIFN-α yield was about 528 μg per larva, showing that the expression in silkworm was successful. Furthermore, the recombinant protein was proven to be able to inhibit the infection of Madin–Darby canine kidney cells by the vesicular stomatitis virus, indicating that it is biologically active in vitro. The method established in this study provides an efficient way to produce a large amount of CaIFN-α and paves the way for further utilization of this protein as a therapeutic agent or vaccine adjuvant in dogs.

Keywords Canine interferon-α · BmNPV/Bac-to-Bac expression system · Silkworm

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Introduction

Along with rapid economic development, raising of pets (particularly dogs) is becoming more popular, especially in developing countries like China. The danger from animal pathogens, particularly viruses, is becoming more serious. For example, human rabies, mainly caused by canine viruses, is a fatal disease. How to prevent and control these infectious diseases is becoming a pressing problem that we have to address. Efficient vaccines or biomedicine may be a good way to solve this problem.

Interferons (IFNs) are a group of immunoregulatory proteins. They are capable of regulating a wide spectrum of biological functions, including immune and inflammatory responses, tissue repair, and antiviral properties. IFN-α is a pleiotropic cytokine that has antiviral, antiproliferative, and immunoregulatory functions (Belardelli et al. 2002; Samuel et al. 2001). Besides its protective role against viral infection and the fact that it is tumorcidal, IFN-α also has biological activities against non-viral pathogens and regulates systemic immune responses (Bogdan 2000; Decker et al. 2005; Ohya et al. 2005). Thus, IFN-α may be effective in a broader range of clinical applications as an adjuvant or immunomodulator in the treatment of a wide range of infectious diseases.

The canine IFN-α (CaIFN-α) has been well studied, and its complementary DNA size is 564 bps, encoding a signal peptide of 23 amino acids and a mature peptide of 164 amino acids. In previous reports, it was expressed and characterized in *Escherichia coli* (Himmler et al. 1987; Taira et al. 2005).

The baculovirus expression system has been proven to be one of the most effective expression tools (Jeefrey et al. 1996; Xiaofeng et al. 2004a, b). The *Bombyx mori* nucleopolyhedrosis virus (BmNPV) expression system can take advantage of the inexpensive, convenient, and high-level production of heterologous proteins in silkworm larvae. We have successfully applied this system to produce large amounts of foreign proteins, such as human vascular endothelial growth factor (Xiaofeng et al. 2004a, b), in silkworm. In previous studies, bovine and canine IFN-γ were reported to be successfully expressed by using a baculovirus system (Giovanna et al. 2006; Fumiyoshi et al. 2000). In this paper, we describe the utilization of a BmNPV/Bac-to-Bac expression system that can generate the recombinant virus rapidly through a bacterial transposon for the production of a large amount of the recombinant CaIFN-α in silkworm.

Materials and methods

Materials

The canine IFN-α gene was cloned from a local dog in Hangzhou, People's Republic of China. As the interferon gene has no intron, it was cloned directly from its genomic DNA.

The DNA extraction kit was purchased from Qiagene. DNA manipulation and polymerase chain reaction (PCR) amplification kits were obtained from Takara Biomedicals. The TA cloning kit, baculovirus donor vector pFastBacHT, lipofectin, and Ni-NTA agarose were purchased from Invitrogen. Fetal calf serum (FCS) and culture medium TC-100 for the cell line BmN were products of GibcoBRL.

The *B. mori* cell line, BmN, was maintained at 27°C with TC-100 medium containing 10% (*v*/*v*) FCS and 0.26% Bacto-tryptose. A hybrid strain of silkworm (commercial name *Baiyu x Qiufeng*) was used in this experiment. The larvae were reared with mulberry leaves at 25–27°C.

Cloning of the canine IFN-α gene and construction of recombinant baculovirus

The CaIFN-α gene was cloned by PCR using dog genome DNA as template. The primers were designed according to the sequence information available in GenBank (accession no. M28624). The sense primer was 5′-*GGATCC*TGC CACCTGCCCGAC-3′ (*Bam*HI), and the antisense primer was 5′-*AAGCTT*TCATTTCCTCCTCCTG-3′ (*Hin*dIII). The PCR result was analyzed by electrophoresis on a 1%-agarose gel (Fig. 1a) and subsequently subcloned into TA cloning vector pGEM® T Vector (Promega). The sequence was confirmed by the dideoxy-chain termination method with a nucleotide sequencer.

For the construction of recombinant baculovirus, the BmNPV/Bac-to-Bac expression system developed recently by our group (Cuiping et al. 2006) was applied. This system can generate recombinant baculovirus genome rapidly in bacteria through gene transposition by transposon Tn7. The CaIFN-α gene was first cloned into the sites of

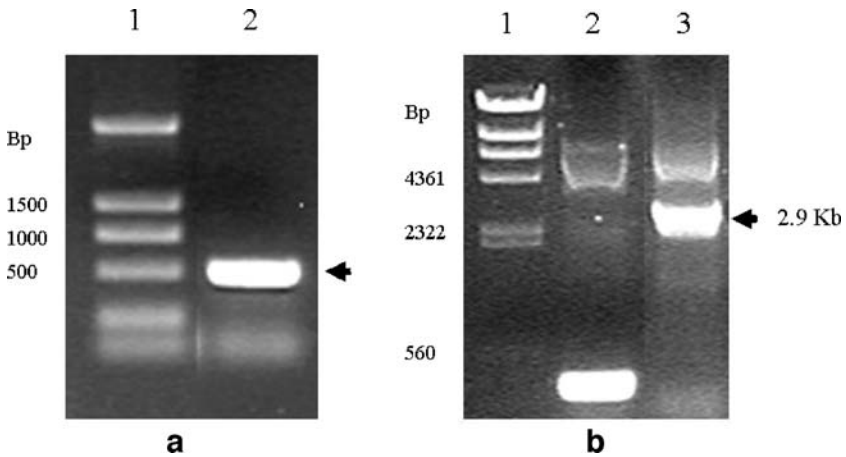

Fig. 1 **a** The PCR result for the canine interferon-α gene. *1* Molecular marker, the size (bp) was listed on the *left*; *2* the cloned fragment of CaIFN-α. Its predicted size was 495 bp. **b** Analysis of recombinant bacmid DNA by PCR to verify the presence of the CaIFN-α gene in the recombinant Bmbacmid by using M13 forward (−40) and M13 reverse primers. The bacmid contained M13 priming sites flanking the mini-*att*Tn7 site within the *lac*Zα complementation region to facilitate PCR analysis (detailed information available at P23, Bac-to-Bac manual by Invitrogen). *1* Molecular marker, the size (bp) was listed on the *left*; *2* negative control. The PCR was performed using the BmBacmid as template, and its predicted size was 387 bp; *3* PCR using recombinant BmBacmid as template. The predicted size was about 2.9 kb

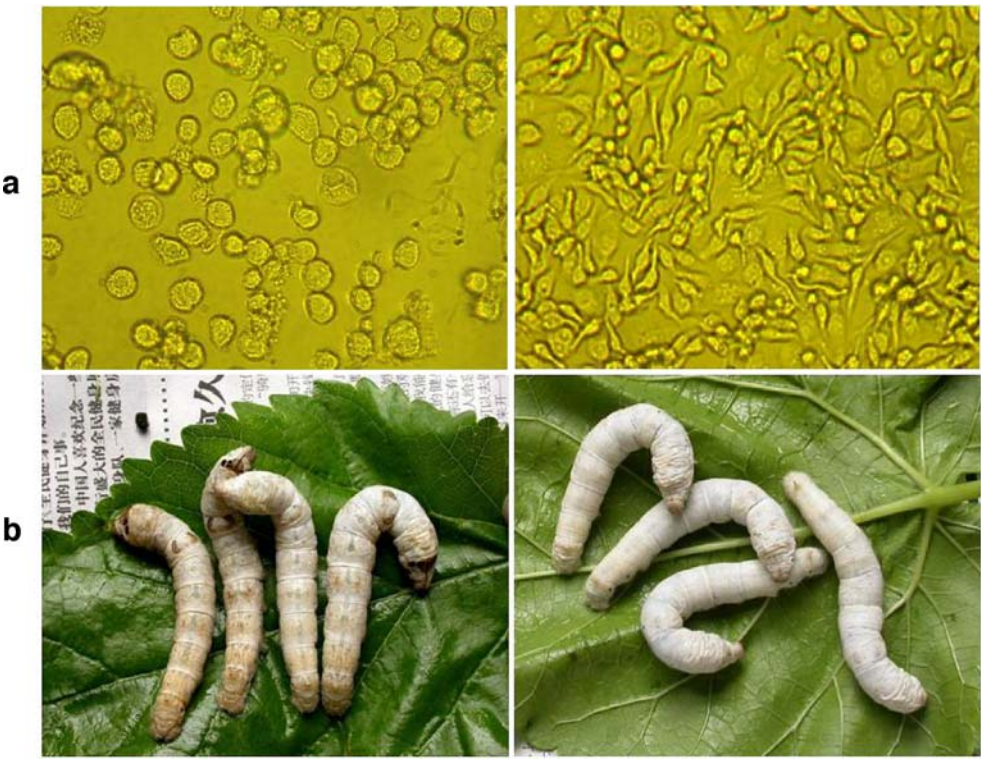

Fig. 2 Test of the infection ability of the recombinant rCaIFN-α baculovirus, with *B. mori* cells and larva. **a** BmN cells. *Left* infected; *right* the control (healthy). **b** The silkworm larva. *Left* infected; *right* the control (without inoculation, healthy)

*Bam*HI/*Hin*dIII in the donor vector pFastBacHTb. The donor contains an expression cassette within the left and right arms of the Tn7 transposon. The recombinant pFastBacHTb–CaIFN-α was subsequently transformed into DH10BmBacmid containing a reconstructed BmNPV genome. About 1 ng of the recombinant donor plasmid was transformed into 100 μl of the competent DH10BmBacmid cells. The mixture was incubated at 37°C for 4 h to achieve transposition. Then, the cells were diluted serially using SOC medium and spread evenly on the plates. The transformants were screened by growth on Luria–Bertani agar plates containing kanamycin (50 μg/ml), tetracycline (7 μg/ml), X-gal (100 μg/ml), and isopropylthiogalactoside (40 μg/ml). After 48 h of incubation at 37°C, the white colonies were selected and cultured overnight in a medium containing kanamycin. The recombinant Bacmid DNA was subsequently extracted and transfected into BmN cells to generate the recombinant baculovirus. Five days later, the medium supernatant was collected as stock virus and used to infect the silkworm larvae.

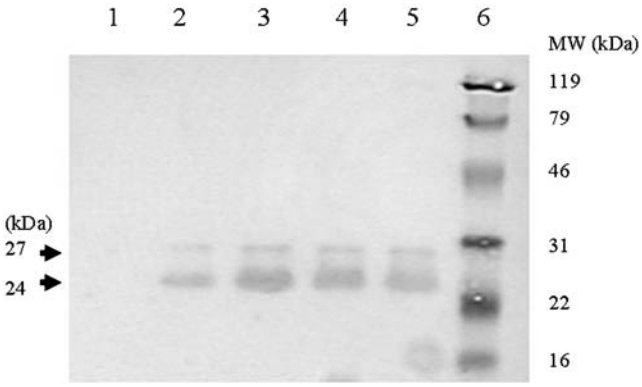

Fig. 3 Time course analysis of the rCaIFN-α expression in the silkworm cell line, BmN cells. The expressed protein was detected by Western blotting analysis. The cells were lysed and subjected to SDS-PAGE (15% gel) and then transferred to polyvinylidene difluoride membrane. The first antibody was a monoclonal antibody, anti-His6 (mAb, *Roche*). *Lanes 1–5* are 0, 48, 72, 96, and 120 h post-infection, respectively; *lane 6* the pre-stained standard, molecular weight marker; their protein sizes are listed on the *left*

Production of canine IFN-α in silkworms and protein purification

The newly molted fifth instar silkworm larvae were used as host for virus infection and expression. So that the larvae did not move actively, they were anesthetized by immersion in a water bath for 10 min before inoculation. About 10^6 plaque-forming units of the recombinant virus (20 μl) were injected subcutaneously into the larvae with a syringe (100 heads/batch). Half an hour after the injection, the larvae were fed with mulberry leaves and then reared at 25–27°C. During the first 3 days, no obvious symptoms were observed. By the fourth day, the larvae began to lose appetite and displayed the infection symptoms. Ninety-six hours after infection, no more feed was given to the larvae to clean their guts, and, subsequently, the larvae were collected.

To increase the production of recombinant protein, the whole bodies of the infected larvae were homogenized with

Table 1 The infection efficiency of silkworm and the protein yield

	Number of larva inoculated	Number of larva infected	Infection rate (%)	Yield obtained from 100 individuals (mg)	Average yield per larva (μg/per larva)
Test	321	306[a]	98.2	52.8	528
Control[b]	200	0	0	0	0

[a] The other 15 larvae died of bacterial contamination because of careless inoculation.
[b] The control group without any treatment

ice-cold extraction buffer (50 mM $NaPO_4$, 0.5 M NaCl, pH 8.0, 1 mM DTT), and then the mixture was centrifuged at 10,000×*g* for 30 min at 4°C to remove large debris and lipids. The supernatant was filtered to remove the remaining lipids, and the filtrate was centrifuged again at 14,000×*g* for 30 min at 4°C. The supernatant was collected as the crude extract of recombinant CaINF-α (rCaIFN-α).

As rCaIFN-α carries a 6× His–tag at the N-terminal, it was purified by using Ni-NTA affinity columns under native conditions. The crude extracts were diluted with native binding buffer (50 mM $NaPO_4$, 0.5 M NaCl, pH 8.0) and then applied directly to Ni-NTA columns for binding. The rCaIFN-α was finally eluted by native elution buffer (binding buffer plus 250 mM imidazole). The purified protein was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Bioassay of recombinant canine IFN-α

The antiviral activity of rCaIFN-α was determined by a cytopathic effect (CPE) inhibition assay, using vesicular stomatitis virus (VSV) on the Madin–Darby canine kidney (MDCK) cell line according to the reported method (Taira et al. 2005). The cells were seeded into 96-well tissue culture plates at a density of about 1.0×10^4 cells/well in a 1-ml volume and cultured at 37°C and 5% CO_2 for a few days until the cells covered the layer evenly. The purified rCaIFN-α, which was diluted serially and filtered through a 0.22-μm sterile filter, was added to the cells. The VSV virus was subsequently added with the dose of $100TCID_{50}$. A negative control was also established. After 24 h, the CPE of cells was observed under a microscope.

Results

The CaIFN-α gene was successfully cloned from our local dog and subsequently analyzed. The detailed information regarding this sequence was registered in GenBank with accession number EF080825. Comparison of this sequence with the previously registered M28624 showed only three nucleotide changes at positions 9 (G-A), 153 (G-T), and 408 (G-A), but the deduced amino acid sequence was identical, implying that the CaIFN-α gene is conserved.

The recombinant baculovirus containing the CaIFN-α gene was quickly generated by our BmNPV/Bac-to-Bac

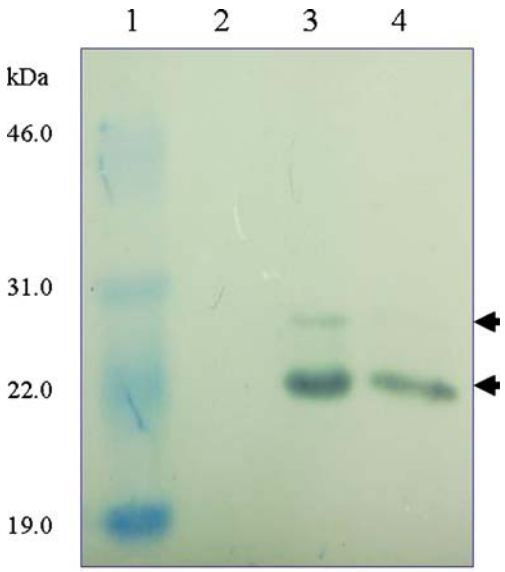

Fig. 4 The purification of expressed rCaIFN-α. The sample was identified by SDS-PAGE (15% gel). *Lane 1* Molecular weight marker, *lane 2* the control without infection, *lane 3* purified rCaIFN-α from the infected silkworm larvae, *lane 4* small-scale purified sample from the infected BmN cells

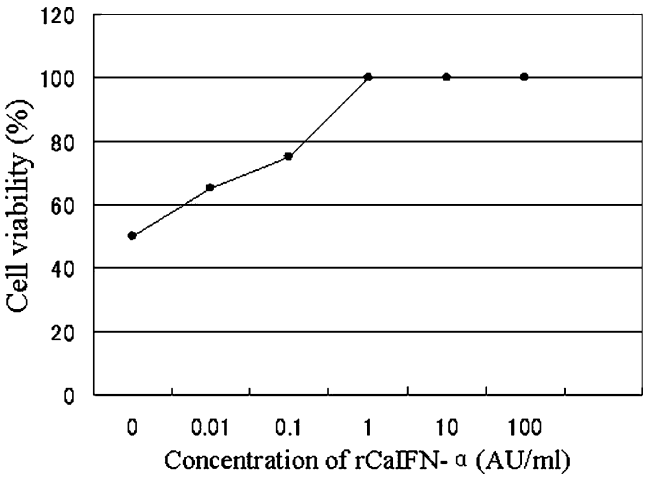

Fig. 5 Antiviral activities of purified rCaIFN-α on MDCK cells. MDCK cells were infected with 1×10^5 of VSV virus in the presence of serial doses of rCaIFN-α. After 24 h, the cell viability was measured (in triplicate) and expressed as mean viability ± standard deviation

expression system. The insert of the CaIFN-α gene into the BmBacmid was confirmed (Fig. 1b). Thus, the obtained recombinant virus can infect the *B. mori* cells and the BmN cell line, as well as the silkworm larvae, very well (Fig. 2). To confirm the expression of the rCaIFN-α and make clear its expression time course, BmN cells were first infected. The culture media and cells were collected and, subsequently, subjected to SDS-PAGE and Western blotting. As shown in Fig. 3, two interesting bands of approximately 27 and 24 kDa, respectively, were observed in the sample of lysed, infected cells (the upper band was very weak, but visible). The time course analysis by Western blotting indicates that the expression level of rCaIFN-α in the cells reached its peak around 72–96 h post-infection.

For larger-scale production, 321 larvae were inoculated. The infection efficiency and the protein yield are summarized in Table 1. The larvae were infected well (98.2%), although some died because of bacterial contamination. One hundred larvae were used for purification, and, finally, about 52.8 mg of rCaIFN-α was obtained, an average of 528 μg per larva. This amount is equal to the production from 52.8×300 ml (15,840 ml) of insect culture supernatant, according to a previous report (Ruttanapumma et al. 2006), indicating that the expression level in silkworm is very high. The purified rCaIFN-α from the larvae was identified by SDS-PAGE and shown to be pure, with two bands (Fig. 4). Based on the deduced amino acid sequence, the molecular weight of the rCaIFN-α in this experiment was calculated to be approximately 22.39 kDa (including six histidines and others from the multiple cloning site, 3.39 kDa). As the CaIFN-α contains two potential glycosylation sites, according to its amino acid sequence, it is possible that the expressed protein is glycosylated. Two bands (27 and 24 kDa) in the purified rCaIFN-α were presumed to be mature forms, glycosylated at two sites or one site, respectively. In a previous report, the rCaIFN-α expressed in *E. coli* was confirmed as consisting of only one band and being homogeneous (Taira et al. 2005). Thus, our results demonstrate that the silkworm–baculovirus expression system has a better post-translational modification capability than the *E. coli* expression system.

The bioassay indicates that the rCaIFN-α expressed in silkworm can inhibit effectively the infection by VSV virus (Fig. 5). The IFN titer, arbitrary units per milliliter (AU/ml), was calculated as a reciprocal of the highest dilution of the wells in which 100% of the monolayer was protected from the CPE of the virus. As shown in Fig. 5, the titer of purified rCaIFN-α was determined to be 1.0×10^7 AU/ml. Thus, the bioactivity of rCaIFN-α on MDCK cells was calculated to be approximately 5.0×10^7 U/mg, indicating that the rCaIFN-α generated from silkworms is biologically active. According to this bioassay data, the specific activity of rCaIFN-α produced in silkworm was about three times higher than that of rCaIFN-α produced in *E. coli* (Taira et al. 2005). Therefore, our data suggest that the antiviral activity of rCaIFN-α from silkworm might be related to the glycosylation of this protein.

Discussion

The silkworm is economically important for silk production in many developing countries. Modern biotechnology has made it an ideal 'biofactory' for producing recombinant protein, including vaccines, as the larva has lots of advantages such as a large body, short lifecycle, easy rearing, good biosafety (the moth loses its ability to fly), and, most importantly, low cost. Most foreign proteins expressed in larvae undergo normal post-translational modifications. However, in some cases, glycosylation is a problem in baculovirus expression systems, especially when expressed in cell line. In this experiment, however, the recombinant CaIFN-α expressed in silkworm was glycosylated. This fact further indicated that the silkworm has better post-modification capacity and the recombinant protein from this insect will be biologically functional.

The method established in this study may be the most economical and efficient way to produce large amounts of biologically active rCaIFN-α. Similarly, it is possible to produce other useful proteins and other vaccines, which could be applied in stock raising, medicine, and agriculture, with silkworms–BmNPV/Bac-to-Bac system. This is particularly significant for developing countries, for the control of many infectious animal diseases.

Acknowledgment This work was supported by the National High-tech R&D Program (2007AA10Z159), Key International Cooperation Programme of Zhejiang (2006C24015), and a grant of the New Century Training Programme Foundation for the Talents (NCET-06-0524), Ministry of Education, China.

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