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# Expression and purification of recombinant feline interferon in the baculovirus-insect larvae system

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ABSTRACT

Feline interferons (FeIFNs) are cytokines with antiviral, antitumor and immunomodulatory functions used as therapeutic agents in a variety of veterinary diseases. In this work, FeIFN-α7 and FeIFN-α7xArg containing eight residues of arginine were expressed in Sf9 cells and insect larvae. At 4 days post-infection (dpi), the concentrations of FeIFN-α7 and FeIFN-α7xArg in suspension culture were $(1.28 \pm 0.15) \times 10^6$ U ml$^{-1}$ and $(1.3 \pm 0.2) \times 10^6$ U ml$^{-1}$ respectively. The maximum expression levels of FeIFN-α7 and FeIFN-α7xArg were $(3.7 \pm 0.2) \times 10^6$ U ml$^{-1}$ and $(3.5 \pm 0.4) \times 10^6$ U ml$^{-1}$ at 2 dpi in *Rachiplusia nu* larvae and $(1.1 \pm 0.2) \times 10^6$ U ml$^{-1}$ and $(1.0 \pm 0.15) \times 10^6$ U ml$^{-1}$ at 5 dpi in *Spodoptera frugiperda* larvae respectively. *R. nu* was a better host for FeIFN-α7 and FeIFN-α7xArg expression. The 8xArg tag did not affect the biological activity of FeIFN-α7 and was useful to promote the FeIFN-α7xArg adsorption on ion exchange chromatography (IEC), allowing its purification in a single step from supernatant culture and *R. nu* larvae. FeIFN-α7xArg was purified from the larval extract with a yield of 70% and a purification factor of 25 free of viruses. We conclude that *R. nu* larvae are new low-cost hosts for the expression of recombinant FeIFN-α7.



## 1. Introduction

Feline interferons (FeIFNs) are cytokines with antiviral, antitumor and immunomodulatory functions used as therapeutic agents in a variety of veterinary diseases [1,2]. Recombinant FeIFN-ω has been expressed in *Bombyx mori* larvae infected with baculovirus and is now available as a commercial product [3–5]. FeIFN-ω has been shown to improve symptoms and prolong survival of animals infected with feline herpesvirus, feline calcivirus, feline peritonitis, feline leukemia and feline immunodeficiency [2,6]. FeIFN-ω has also been tested in the treatment of feline and canine neoplasms [7,8] and of viral diseases in dogs [9], with good results. FeIFN-ω has 99% identity to FeIFN-α7, differing by only one nucleotide belonging to a signal peptide. FeIFN-α7 has been cloned from a feline epithelial cell line and expressed and characterized in *Escherichia coli* [10]. FeIFN-α7 has a molecular weight of 25 kDa, an isoelectric point (pI) of 6.5 and a potential N-glycosylation site located at position 79–81 [4].

Nowadays, the biotechnology industry demands fast, efficient and economic processes for the expression and purification of biomolecules. For this purpose, the baculovirus system is interesting to produce recombinant proteins, especially for veterinary applications. Insect cell lines such as those from *Spodoptera frugiperda* (Sf21, Sf9) and *Trichoplusia ni* (Tn-5) are widely used because of their susceptibility to *Autographa californica* nucleopolyhedrovirus (AcMNPV), the baculovirus expression vector most commonly used. This system has been extensively used for the production of several recombinant interferons (INFs) [11–16]. However, tissue culture techniques are expensive because they need large quantities of culture medium and sophisticated equipment. Thus, the scaling-up of protein production using insect larvae (Lepidoptera: Noctuidae) as biofactories has become an attractive strategy to explore because it is simpler, more inexpensive and less

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time-consuming than insect cell cultures, and has greater efficiency in post-translational modifications and lower contamination risk [17]. Insect larvae other than *B. mori* larvae are not widely exploited mainly because of the lack of experience in rearing and maintaining them in laboratories and the limited knowledge of purification processes [18–20]. *B. mori* is a very efficient host but the main value of this larva is in the silk industry. In contrast with *B. mori, Rachiplusia nu, S. frugiperda* and other lepidopteran species are plagues without an economic value. For this reason, it is interesting to explore these larvae as alternative hosts to produce recombinant proteins. *S. frugiperda* and *R. nu* are some of the most abundant and widely distributed lepidopteran plague species in South America. Both species are hosts permissive to AcMNPV infection when budded virus is injected intrahemocelically [21,22]. In our laboratory, we have studied the use as hosts of different lepidopteran species frequently found in Argentina, and successfully expressed the horseradish peroxidase isozyme C (HRP C) with similar levels in *S. frugiperda* and *R. nu* [23].

On the other hand, most recombinant IFNs expressed in the baculovirus system have been usually purified by affinity chromatography using metal ions or triazine dyes [4,24,25]. In contrast with affinity chromatography, ion exchange chromatography (IEC) not only meets cGMP requirements but also has vast advantages as easy and linear scalability and low cost. We have previously reported that the addition of a 6xArg fusion tag to horseradish peroxidase isozyme C (HRP C) increases its pI and facilitates its purification by IEC from baculovirus culture medium [26].

In this study we explored different strategies to achieve high levels of expression of recombinant FeIFN-α7 and facilitate its purification by IEC from cell cultures and insect larvae.

## 2. Materials and methods

### 2.1. FeIFN-α7 cDNA sequence

The full-length FeIFN-α7 sequence (NM_001009197) was obtained from the National Center for Biotechnology Information data bank. cDNA was synthesized by GenScript (Piscataway, NJ, USA) and cloned in the pUC18 plasmid. The synthetic gene does not include the endogenous 5′leader sequence of the feline gene. The pUC18 containing the full-length gene was amplified in *E. coli* DH5α under ampicillin selection and purified by AxyPrep Miniprep Kit (Axygen Biosciences, Union city, CA, USA). Then, it was digested with EcoRI and BamHI restriction endonucleases (Promega, Madison, WI, USA) to obtain the FeIFN-α7-encoding fragment.

### 2.2. Construction of transfer vectors

Two different expression cassettes were constructed. For the first construction, the FeIFN-α7 gene was directly cloned into the pAcGP67-B transfer vector (BD Biosciences Pharmingen, San Diego, CA, USA) downstream the baculovirus polyhedrin promoter and the gp67 viral signal peptide sequence which target the recombinant protein for secretion (pAcFeIFN-α7 construction). For the second (pAcFeIFN-α7xArg) construction, the stop codon of the FeIFN-α7 gene was removed by PCR using two primers designed in our laboratory: Forward (5′-3′): CAGGATCCTGTGACCTGCCTCAGACC; Reverse (5′-3′): CGAATTCCTTTCTCGCTCCTTAATCTTTTCTGC. PCR conditions (50 μl final volume) were: 0.2 M each primer, 1× PFU buffer, 0.3 mM each dNTP and 2.5 U PFU DNA polymerase (Promega). The PCR program was: a first step of 6 min at 95 °C, 29 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min. An additional extension step of 7 min at 72 °C was then applied.

After the reaction, free primers from the PCR product were removed by using the PCR WizardTM SV gel and PCR Clean-up System (Promega).

On the other hand, the short DNA fragment encoding eight arginine residues was generated by annealing and primer extension with Klenow DNA polymerase (Promega). This sequence was inserted in the pAcGP67-B transfer vector (BD Biosciences Pharmingen) for achieving a fusion protein with higher pI.

The following primers were designed to generate the short fragment: Forward (5′-3′):

ATGAAATTCGTCGTCGTCGTCGTCGTCGT; Reverse (5′-3′):

TCAAGTCTAACGACGACGACGACGACGACGAA. The primer extension conditions (20 μl final volume) were: 0.2 M each primer, 1× Klenow buffer, 125 mM of each dNTP and 2.5 U Klenow DNA polymerase. The primer extension program was: 70 °C for 5 min, 40 °C for 5 min, 25 °C for 30 min, and 70 °C for 10 min. The duplex after the PCR reaction was digested with EcoRI and BamH1 restriction endonucleases (Promega). The fragment was purified by two phenol-chloroform extractions and precipitated by one volume of ammonium acetate 7.5 M and two volumes of ethanol 100%. Finally, the fragment was resuspended in 25 μl of Tris–HCl 10 mM pH 8.0 and cloned into the pAcGP67-B transfer vector to obtain a new vector named pAcGP67xArg.

The FeIFN-α7 gene lacking the stop codon and the pAcGP67xArg vector were digested with EcoRI and BamHI restriction endonucleases and ligated to obtain the pAcFeIFN-α7xArg construction. FeIFN-α7 was cloned downstream the baculovirus polyhedrin promoter and the gp67 viral signal peptide sequence.

### 2.3. Virus production

One million Sf9 cells were co-transfected with 1 μg of each construction and 250 ng linearized Baculo Gold Bright DNA (BD Biosciences Pharmingen) in the presence of Cellfectin (Invitrogen Life Technologies, Gaithersburg, MD, USA). Baculo Gold Bright DNA contains the gene for green fluorescent protein (GFP). After a 4-day incubation at 27 °C, the cell culture supernatant was collected and centrifuged at 3000 × *g* for 10 min. Co-transfection efficiency was determined by measuring GFP expression by fluorescence under UV light. Fig. 1 shows both cloned fragments FeIFN-α7(70-585) and FeIFN-α7(70-582) into both polyhedrin-minus AcFeIFN-α7 and AcFeIFN-α7xArg respectively. The viable viruses present in the co-transfection supernatant were recombinant because the viral genome had a lethal deletion and only by homologous recombination with the recombinant transfer vector this deletion was recovered. So, after homologous recombination it was not necessary a step of virus purification from supernatant. The co-transfection supernatant was used to infect monolayer cultures at a multiplicity of infection (MOI) of 0.02 (first amplification). After 4-day of incubation at 27 °C, the cell culture supernatant was collected again. This first amplification supernatant was used to perform the second amplification and so on. Following three amplification steps, the virus titer was determined by a plaque assay [27]. The amplified virus stock was used at the production step.

### 2.4. Insect cell infection

Sf9 cell suspension cultures (Invitrogen Life Technologies) were grown in sterile Erlenmeyer flasks under continuous shaking at 100 rpm in Sf900II medium supplemented with 1% (v/v) fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Invitrogen Life Technologies) at 27 °C. The culture volume did not exceed 10% of the total volume of the Erlenmeyer flask.

Recombinant FeIFN-α7 and FeIFN-α7xArg were produced in suspension cultures of 10 ml in Erlenmeyer flasks in log-phase at a cell density of $1 \times 10^6$ cells $ml^{-1}$ (95–99% viability). The cultures