Process Biochemistry 49 (2014) 917–926

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

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

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ARTICLE INFO

Article history:
Received 15 December 2013
Received in revised form 8 February 2014
Accepted 11 March 2014
Available online 25 March 2014

Keywords:
Feline interferon
Expression
Baculovirus
Purification
Polyarginine tag
Ion exchange chromatography

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.

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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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http://dx.doi.org/10.1016/j.procbio.2014.03.013
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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′):

ATGAAATTCGTCGTCGTCGTCGTCGTCGTCGT; Reverse (5′-3′):

TCAAGTCTAACGACGACGACGACGACGACGACGAA. 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 1×10^6 cells ml^{-1} (95–99% viability). The cultures

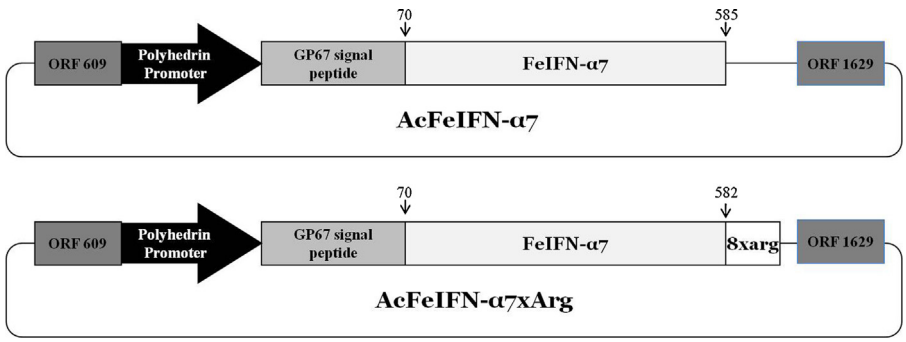

Fig. 1. Recombinant baculovirus polyhedrin-minus constructions (AcFeIFN-α7 and AcFeIFN-α7xArg). The synthesized FeIFN-α7 (70-585) and FeIFN-α7 (70-582) genes were fused in-frame to the viral signal peptide GP67 and were under control of the polyhedrin promoter. ORF (open reading frame).

were infected in triplicate at MOI of 1, 2 and 5. An uninfected cell culture and a cell culture infected with baculovirus AcGP67HRPC -previously constructed in our laboratory- were also included as control experiments [17]. Infected cultures were incubated in an orbital shaker at 100 rpm at 27 °C for 6 days. The FeIFN-α7 and FeIFN-α7xArg expression in supernatants was determined every day post-infection (dpi). The cells were removed by centrifugation (3000 × *g*, 10 min) and biological activity was measured in the supernatants. The results are expressed as the average ± standard deviation of at least three determinations.

2.5. Larval infection

R. nu larvae (Agidea, Pergamino) and *S. frugiperda* larvae (INTA Sáenz Peña) were obtained from a laboratory colony fed on a high-wheat germ diet [28]. Second-instar larvae were reared in 6-well dishes (one per well) at 24 °C in a 70% humidified chamber, with a 16:8 photoperiod (L:D).

For FeIFN-α7 and FeIFN-α7xArg expression kinetics, 50 fifth-instar larvae (20 days of age) were used for each condition. For all experiments, fifth-instar larvae were sedated by incubation on ice for 5 min and then injected with 7×10^5 PFU of each recombinant baculovirus in independent experiments near the third prolegs. Uninfected larvae or larvae infected with baculovirus control AcGP67HRPC were included as controls [17]. A group of infected larvae were kept at 24 °C and another at 27 °C. Insect infection was followed until the death of the larva. Only living larvae were harvested for interferon determination. After the allotted time, larvae were harvested and homogenized in groups of five larvae ($n = 10$) in the presence of 1 ml buffer lysis per larva (50 mM sodium phosphate buffer, pH 6.0, 5 mM EDTA, 0.2 mg ml^{-1} PMSF, 150 mM KCl with glutathione crystals and protease inhibitor cocktail (Sigma–Aldrich, Saint Louis, MO, USA) per gram of larvae to obtain larval extracts. Each extract was centrifuged at 14,000 × *g* for 10 min at 4 °C. FeIFN-α7 and FeIFN-α7xArg antiviral activity in the extract was measured and results are expressed as the average ± standard deviation.

2.6. In vitro viral inhibition assay

The antiviral activity of FeIFN-α7 and FeIFN-α7xArg was determined by the 50% cytopathic effect reduction assay, using vesicular stomatitis virus (VSV, ATCC VR-158, Indiana Strain, Manassas, VA, USA) and Crandell feline kidney (CRFK, ATCC CCL-94, Manassas) cells [29]. CRFK cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 with modified Eagle's medium supplemented with 10% FBS and antibiotics (Invitrogen Life Technologies). The cells were seeded into 96-well tissue culture plates (5×10^4 cells/well) in 100 μl volume and incubated for 24 h with 100 μl of two-fold serial dilutions of samples from baculovirus-infected culture supernatant, larval extract and purified product containing FeIFN-α7 or FeIFN-α7xArg. Samples were previously filtered through a 0.22-μm membrane. Untreated cells and cells treated with FeIFN-ω (Virbac, France) as standard (1.6×10^8 U mg^{-1}) were also included as controls. Cells were incubated for 24 h at 37 °C and 5% CO_2. Then, the cells were infected with 100 μl of VSV at a MOI of 0.25. A non-infected control was also included. After 24 h, cells were fixed in 5% (v/v) formalin and stained with 0.1% (w/v) crystal violet. Then the cells were washed with deionized water and the cytopathic effect was measured in an ELISA reader at 595 nm. Antiviral activity was calculated by units (U) from a standard curve using the FeIFN-ω. The results are expressed as the average ± standard deviation of at least three determinations.

2.7. In vitro cytotoxic activity on feline mammary carcinoma cells

Feline mammary carcinoma cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 with Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 supplemented with 10% FBS, 10 mM HEPES (pH 7.4) and antibiotics (Invitrogen Life Technologies). The cells were seeded into 96-well tissue culture plates (4×10^4 cells/well) in 100 μl volume and incubated as mentioned above for 5 days with serial 2-fold dilutions of the samples from baculovirus-infected culture supernatant, larval extract and purified product containing FeIFN-α7 or FeIFN-α7xArg. Samples were previously filtered through a 0.22-μm membrane. After that, cell viability was evaluated with the Aqueous Nonradioactive Cell Proliferation Assay MTS (Promega). Then, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was added directly to the sample and reduced by metabolically active cells (dehydrogenase activity) to aqueous soluble formazan. The amount of formazan measured at 490 nm is directly proportional to the number of viable cells in the sample. The results are expressed as the average ± standard deviation of at least three determinations.

2.8. Purification of FeIFN-α7 and FeIFN-α7xArg from the culture supernatant by IEC

The samples were conditioned in equilibration buffer (Tris–HCl 5 mM, pH 8.0) by using a PD-10 column (GE Healthcare, Piscataway, NJ, USA). The samples were loaded on a 1.0-ml bed volume SP-HiTrap column (GE Healthcare). After a washing step with equilibration buffer, elution steps were carried out by addition of 60 mM, 350 mM and 1 M NaCl in equilibration buffer. Protein separation was monitored by absorbance at 280 nm. The linear flow rate was 0.4 cm min^{-1}.

2.9. Purification of FeIFN-α7xArg from larval crude extract by IEC

Six larvae were homogenized directly in 6 ml of 5 mM Tris–HCl buffer, pH 8.0 (equilibration buffer) with glutathione crystals and protease inhibitor. The crude extract was centrifuged at 14,000 × g for 10 min at 4 °C and then filtered using a Whatman filter paper grade 1. The ionic strength of the sample was measured with a conductivity monitor (Pharmacia Biotech, Uppsala, Sweden).

The sample was loaded on a SP-HiTrap column (1.0-ml bed volume). After a washing step with equilibration buffer, elution steps were carried out by addition of 30 mM, 150 mM and 1 M NaCl in equilibration buffer. Protein separation was monitored by absorbance at 280 nm. The linear flow rate was 0.4 cm min^{-1}.

2.10. Total protein measurement

Protein concentration was measured using the Bradford assay [30] with bovine serum albumin as standard.

2.11. Electrophoretic analyses

Protein samples were separated by SDS-PAGE on 12.5% polyacrylamide gels and stained with Coomassie Blue. For western blot analysis, the immunogenic peptide SCQKDRNDFAFPQDV was synthesized by GenScript. Mice were immunized with this peptide in the same laboratory (GenScript) to produce polyclonal anti-FeIFN-α7 primary antibody. Gels were transferred onto a nitrocellulose membrane and FeIFN-α7 and FeIFN-α7xArg were detected using the mouse polyclonal anti-FeIFN-α7 as the primary antibody and HRP-conjugated anti-mouse IgG (DAKO, Copenhagen, Denmark) as the secondary antibody. For 3,3′-diaminobenzidine (DAB) staining, the gel was immersed in a 9 mg ml^{-1} DAB aqueous solution (Sigma) with 10 μl of 2.6 M hydrogen peroxide for 2 min. Page ruler prestained protein ladder (Thermo Scientific, Rockford, IL USA) was used as the molecular weight marker.

2.12. Isoelectrofocusing (IEF)

IEF gels (3–9) and the blot to nitrocellulose membrane were performed in the equipment Phast System (GE Healthcare). A standard of pI 5–9 was used. The western blot was revealed with specific anti-FeIFN-α7 as described in electrophoretic analyses.

2.13. Localization of viral particles in the chromatographic fractions by western Blot and PCR

The chromatographic fractions were run on a polyacrylamide gel, electroblotted to nitrocellulose membranes as described above and revealed with a polyclonal primary antibody obtained in mice directed against the viral envelope protein gp67 and HRP-conjugated anti-mouse IgG (DAKO) as the secondary antibody.

On the other hand, viral DNA was purified from the chromatographic fractions using the commercial kit Viral Nucleic Acid Extraction Kit (RBC, Real Genomics, Banquiao, Taiwan) for purification of viral DNA. Subsequently, a PCR was performed with specific oligonucleotides as described in construction of transfer vectors. The PCR reaction was performed as described above.

3. Results and discussion

3.1. Expression of FeIFN and FeIFNxArg in the Sf9 cell line

Sf9 insect cells cultured in SF900II medium containing 1% (v/v) FBS were infected with recombinant baculoviruses (AcFeIFN-α7 and AcFeIFN-α7xArg) in different experiments. The cell line was adapted to grow in serum free media but we observed that the cell growth rate was higher with the addition of 1% fetal bovine serum. Moreover, under the last condition, the expression levels of FeIFN-α7 and FeIFN-α7xArg increased ten-times (data not shown).

The expression kinetic curves based on antiviral activity of both FeIFN-α7 and FeIFN-α7xArg at a MOI of 1 were similar, thus indicating that 8xArg tag did not affect the antiviral activity. Infection at higher MOIs (2, 5) did not increase the expression level of both recombinant proteins (data not shown). The level expression based on antiviral activity gradually increased achieving its maximum at 4 dpi (Fig. 2A). However, from 6 dpi it began to decline in comparison to the 4 and 5 dpi. No activity was detected in negative control experiments. In this work, the recombinant interferon was secreted into the supernatant of expression cultures. Despite of the major drawback at advanced times of infection is the protease activity in supernatants, in this case no degradation was observed as judged by western blot. Therefore, the decrease of antiviral activity at an advanced time of infection could be caused by some grade of instability proper of this molecule [31]. FeIFN-α7 and FeIFN-α7xArg showed a molecular weight of around 25 kDa, as judged by western blot analysis. However, the recombinant products are not completely homogeneous, suggesting that glycosylation forms of the cytokine were present (Fig. 2B and C). Glycosylation is essential for cytokines because it confers greater stability, interaction with specific receptors and pharmacokinetic profile [32,33]. Na et al. have described the expression of recombinant interferon in insect cells and larvae as multiple bands due to glycosylation [25]. Proteins like FGF1, IGFBP6, FGF7 and mouse IFNβ, expressed in Sf9 cells and *B. mori* larvae, have also been detected as double or multiple bands [34]. On the other hand, the smaller band (around 21 kDa) found from 5 dpi could be also explained by the presence of an unglycosylated interferon with unprocessed signal peptide that was released from the cell because of the lysis. However, at 4 dpi the insect cell lysis is not so high (15%) to recover a high amount of immature recombinant protein from the reticulum. Taking this into account, in this work FeIFN-α7 and FeIFN-α7xArg were harvested at 4 dpi from a cell culture infected at a MOI of 1.

The maximum expression levels of FeIFN-α7 and FeIFN-α7xArg reached in suspension culture were $(1.28 \pm 0.15) \times 10^6$ U ml^{-1} (8.0 ± 0.9 μg ml^{-1}) and $(1.3 \pm 0.2) \times 10^6$ U ml^{-1} (8.1 ± 1.2 μg ml^{-1}) respectively, which represents 1.1% of the total protein of the supernatant. This expression is comparable with that of FeIFN-ω obtained by Sakurai et al. in BM-5 cells infected at a MOI of 10. However, these authors also reported that FeIFN-ω expressed in culture medium of BM-5 cells – a *B. mori*-derived cell line- has some degree of degradation because of protease activity. As a consequence, antiviral activity decreased at 4 dpi [4]. In contrast with these results, here we achieved the same expression level of FeIFN-α7 and FeIFN-α7xArg at 4 dpi by using a MOI of 1 instead of a MOI of 10 in the Sf9 cell line.

3.2. Expression of FeIFN-α7 and FeIFN-α7xArg in insect larvae

We then aimed to express FeIFN-α7 and FeIFN-α7xArg directly in *R. nu* larvae (approximately 200 mg each larva) and *S. frugiperda* (approximately 300 mg each larva). To assess the expression kinetics, the antiviral activity of FeIFN-α7 and FeIFN-α7xArg in total larval extracts was measured at different dpi. Larvae were infected with 7×10^5 PFU of AcFeIFN-α7 and AcFeIFN-α7xArg in different experiments and maintained at 24 °C and 27 °C. The best temperature to rear insect larvae is 24 °C, but 27 °C is the best for baculovirus replication [27]. For this reason, we tested the expression experiment at these both temperatures. Besides, we observed that with an inoculum of 3×10^5 PFU the yield of the recombinant proteins decreased by 50% and the larvae pupated during the course of infection. On the other hand, higher inocula (1×10^6 PFU) were lethal in 12 h and no production of

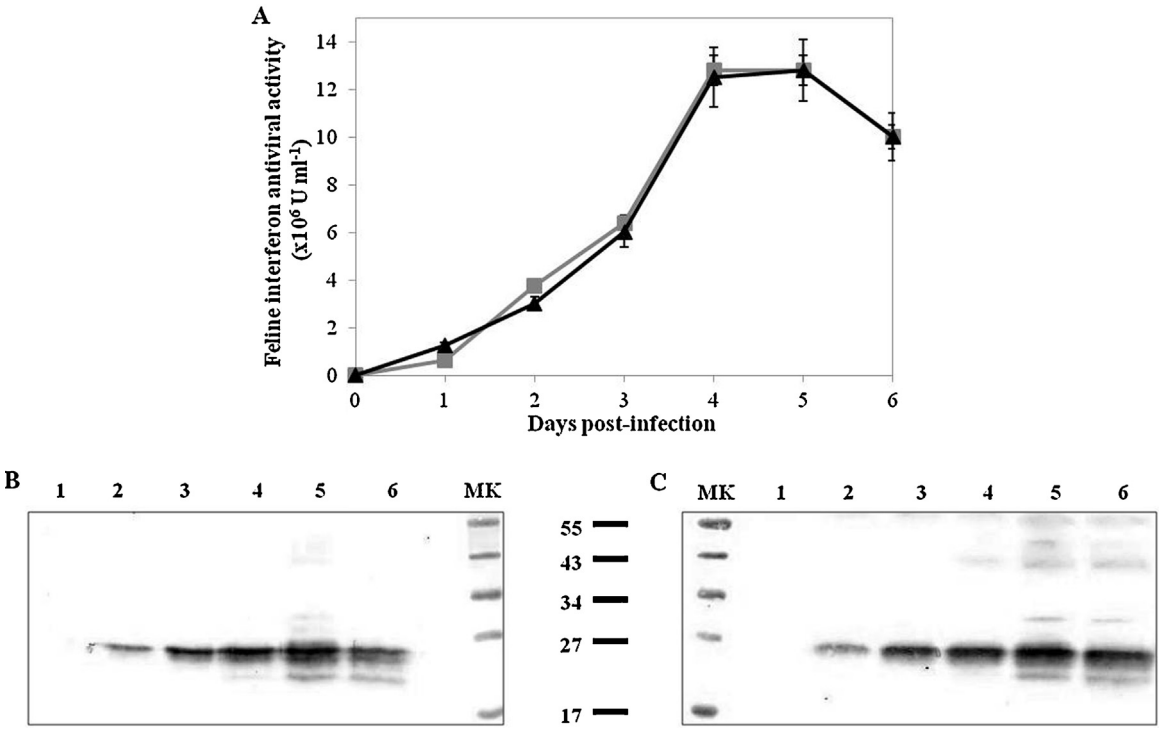

Fig. 2. Time-course analysis of FeIFN-α7 and FeIFN-α7xArg expression in Sf9 cells infected at MOI 1. (A) Expression kinetics of FeIFN-α7 and FeIFN-α7xArg in supernatants of Sf9 cell cultures. (■) FeIFN-α7; (▲) FeINF-α7xArg. Results are expressed as the average ± standard deviation ($n = 3$). (B) Western blot analysis of the expression kinetics of FeIFN-α7. (C) Western blot analysis of the expression kinetics of FeIFN-α7xArg. MK: molecular-weight markers. 1–6: days 1–6 post-infection. The western blot was developed with a specific antiserum raised against a peptide from FeIFN-α7.

recombinant protein was observed. Larvae showed typical symptoms of viral infection such as appetite and mobility loss. *R. nu* infected showed the same expression profile for FeIFN-α7 and FeIFN-α7xArg (Fig. 3A). In all larvae infected and kept 24 °C, the FeIFN-α7 and FeIFN-α7xArg were detected from 3 dpi. Results indicated that the expression greatly increases at the 4 dpi. Day 4 was selected as the optimal condition for the harvest because after that day over 80% of larval mortality was observed (data not shown). Infected larvae maintained at 24 °C showed a maximum expression level of $(3.7 \pm 0.2) \times 10^6$ U ml^{-1} (116.0 ± 6.3 μg g^{-1} larvae) and $(3.5 \pm 0.4) \times 10^6$ U ml^{-1} (110 ± 12.5 μg g^{-1} larvae) for FeIFN-α7 and FeIFN-α7xArg respectively, approximately 0.34% of the total larval proteins. This result suggests again that 8xArg tag had no effect on the antiviral activity of FeIFN-α7. However, in all *R. nu* larvae infected and maintained at 27 °C, the FeIFN-α7 and FeIFN-α7xArg were detected from 1 dpi. Results indicated that the expression greatly increases at 2 dpi. Due to larval mortality after that day (over 80%, data not shown), day 2 was selected as the optimal condition for the harvest. We observed that *R. nu* larvae maintained at 27 °C and harvested at 2 dpi yield a similar expression level than those maintained at 24 °C and harvested at 4 dpi. The temperature strongly influenced the viral replication rate and larval susceptibility to pathogen infection [35–37]. In this case, the high temperature allowed accelerate the expression kinetics due to 27 °C was the best temperature for baculovirus replication. No antiviral activity was observed in negative control experiments. FeIFN-α7 and FeIFN-α7xArg from *R. nu* extract showed a molecular weight of around 25 kDa, as judged by western blot analysis. However, the recombinant products are not completely homogeneous, suggesting that glycosylation forms of the cytokine were present. Previously, it was described for other proteins expressed in this system [34]. Also, the smaller band (around 21 kDa) could be an unglycosylated interferon with unprocessed signal peptide that was released from the cells because of the lysis at advance time of infection (Fig. 4). On the other hand, *S. frugiperda* infected and maintained at 24 °C showed the same expression profile for FeIFN-α7 and FeIFN-α7xArg (Fig. 3B). In all larvae infected and maintained at 24 °C, the FeIFN-α7 and FeIFN-α7xArg were detected from 4 dpi. Results indicated that the expression greatly increases at 6 dpi. Due to larval mortality after this day (over 14%, data not shown), 6 dpi was selected as the optimal condition for harvest. The maximum expression levels were $(1.1 \pm 0.2) \times 10^6$ U ml^{-1} (22.0 ± 4.2 μg g^{-1} larvae) and $(1.0 \pm 0.2) \times 10^6$ U ml^{-1} (20 ± 4.2 μg g^{-1} larvae) for FeIFN-α7 and FeIFN-α7xArg respectively. No antiviral activity was observed in negative control experiments. However, in all *S. frugiperda* larvae infected and maintained at 27 °C, the FeIFN-α7 and FeIFN-α7xArg were detected from 3 dpi. Results indicated that the expression greatly increases at 5 dpi. Due to larval mortality after this day (over 80%, data not shown), 5 dpi was selected as the optimal condition for harvest. *S. frugiperda* larvae harvested at 5 dpi and maintained at 27 °C yield a similar expression level as larvae harvested at 6 dpi and maintained at 24 °C thus demonstrated again the influence of the temperature on the expression level.

S. frugiperda demonstrated slower expression kinetics and lower expression levels (3.4 times less) than *R. nu*. In previous works, we have also reported this difference in the kinetics: we have previously found that the maximum level of recombinant HRP C expression was at 3–4 dpi in *R. nu* larvae and at 6 dpi in *S. frugiperda* [23,38].

While we have previously been able to express HRPC successfully in *S. frugiperda* and *R. nu* with similar levels [23], here *R. nu* was the best host for FeIFN-α7 and FeIFN-α7xArg production. Therefore, *R. nu*, maintained at 27 °C and harvested at 2 dpi, was selected to continue with the expression and purification

Table 1
Comparative analysis of FeIFN-α7 and FeIFN-α7xArg expressed in *Rachiplusia nu* larvae and *Spodoptera frugiperda* larvae.

Larvae	Protein	$\times 10^6$ U ml^{-1}	μg g^{-1} larvae	μg larvae^{-1}	Dpi
R. nu	FeIFN-α7	3.7 ± 0.2	116 ± 6.3	23.2	2
R. nu	FeIFN-α7xArg	3.5 ± 0.4	110 ± 12.5	22	2
S. frugiperda	FeIFN-α7	1.1 ± 0.2	22 ± 4.2	6.6	5
S. frugiperda	FeIFN-α7xArg	1.0 ± 0.2	20 ± 4.2	6	5

experiments. Table 1 shows a comparison of the expression level of FeIFN-α7 and FeIFN-α7xArg reached in *R. nu* and *S. frugiperda* larvae.

3.3. Purification of rFeIFN-α7 and rFeIFN-α7xArg by IEC

The addition of 8xArg tag residues at the C-terminus of FeIFN-α7 expressed in Sf9 cells or *R. nu* larvae increased the pI from 6.5 to 9.15 (Fig. 5). The IEF results showed that most of the contaminating proteins present in the supernatants of Sf9 cultures and total extract *R. nu* larvae have an pI lower than 7.0.

To assess the influence of the 8xArg tag on the chromatographic behavior of the product, IEC purification was performed with FeIFN-α7 and FeIFN-α7xArg expressed in Sf9 cells at pH 8.0. FeIFN-α7xArg (pI 9.15) was adsorbed on the matrix and recovered by increasing the ionic strength to 350 mM NaCl (Fig. 6A). FeIFN-α7xArg was collected in the elution fraction with a yield of 80%, a purification factor of 60 and a specific activity of 1.8×10^8 U mg^{-1}. In contrast, when FeIFN-α7 (pI 6.5) was loaded on the IEC matrix in the same conditions, the protein was found in the pass-through and then gradually lost with successive washing steps (Fig. 6B). These results demonstrate that the presence of 8xArg tag highly influenced the chromatographic behavior.

Based on these results, we assessed the chromatography method with FeIFN-α7xArg expressed in *R. nu* larvae, the best host for FeIFN-α7xArg production. After loading the extract sample, a washing step with 30 mM NaCl was included in this case. After that, FeIFN-α7xArg was eluted by addition of 150 mM NaCl (Fig. 7). In the elution fraction, we obtained FeIFN-α7xArg with a yield of 70%, a purification factor of 25 and specific activity of 1.7×10^7 U mg^{-1}. Although most of the larval proteins have acid pI, 2.5% of the total proteins co-eluted with FeIFN-α7xArg. Thirty % of the FeIFN-α7xArg was not adsorbed on the chromatographic matrix and was lost in the flow-through and washing step. The macromolecules present in the extract, such as contaminant proteins, lipids and viral particles compete with the recombinant protein for the charged ligands of the matrix or impair the interaction between interferon and the charged groups of the matrix. This is frequently seen with complex samples.

The recombinant baculovirus was present in the pass-through and no viral particles were detected by western blot and PCR in the elution fraction containing FeIFN-α7xArg from Sf9 cells (Fig. 8A

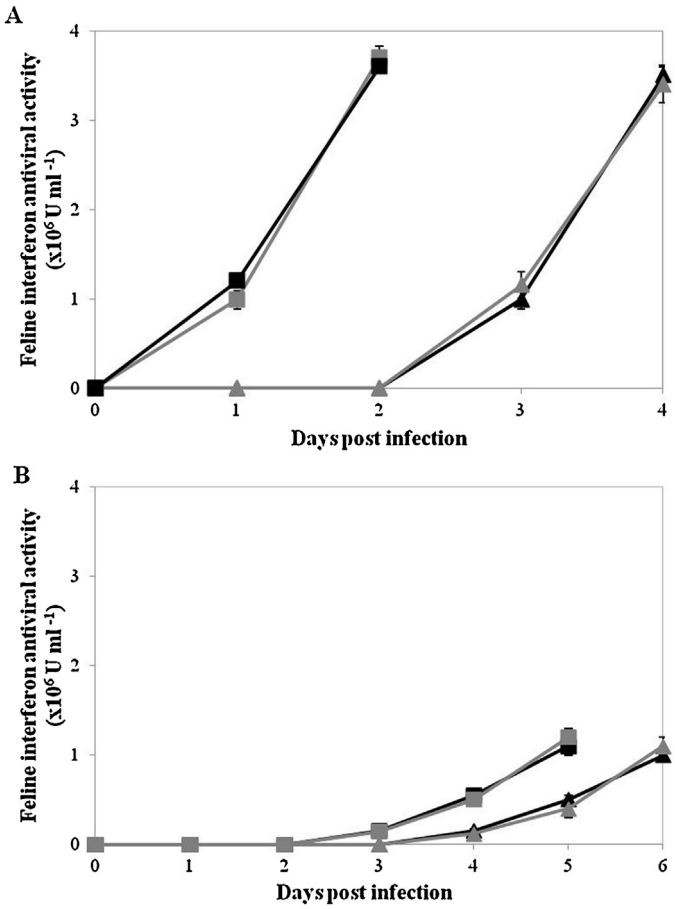

Fig. 3. FeIFN-α7 and FeIFN-α7xArg expression level at different times post-infection in total extract of *R. nu* (A) and *S. frugiperda* larvae (B) at 24 °C or 27 °C. (▲) FeIFN-α7 expressed at 24 °C, (▲) FeIFN-α7xArg expressed at 24 °C, (■) FeIFN-α7 expressed at 27 °C, (■) FeIFN-α7xArg expressed at 27 °C. Results are expressed as the average ± standard deviation (n = 10).

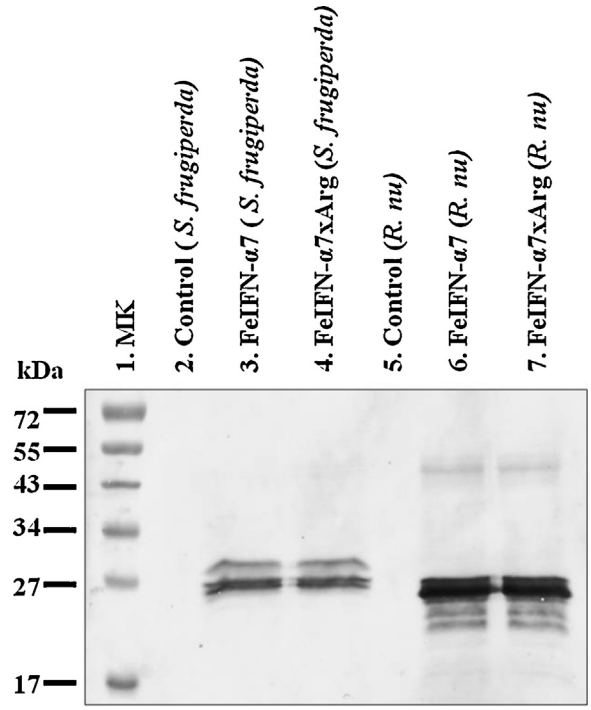

Fig. 4. Expression of FeIFN-α7 and FeIFN-α7xArg in total extract of *R. nu* and *S. frugiperda* larvae infected at 27 °C. Lane 1, molecular-weight marker (MK); lane 2, *S. frugiperda* infected with baculovirus control AcGP67HRPC (Control); lane 3, FeIFN-α7 from *S. frugiperda*; lane 4, FeIFN-α7xArg from *S. frugiperda*; lane 5, *R. nu* infected with baculovirus control AcGP67HRPC (Control); lane 6, FeIFN-α7 from *R. nu*; lane 7, FeIFN-α7xArg from *R. nu*. Western blot was developed with specific polyclonal antiserum raised against a peptide from FeIFN-α7.

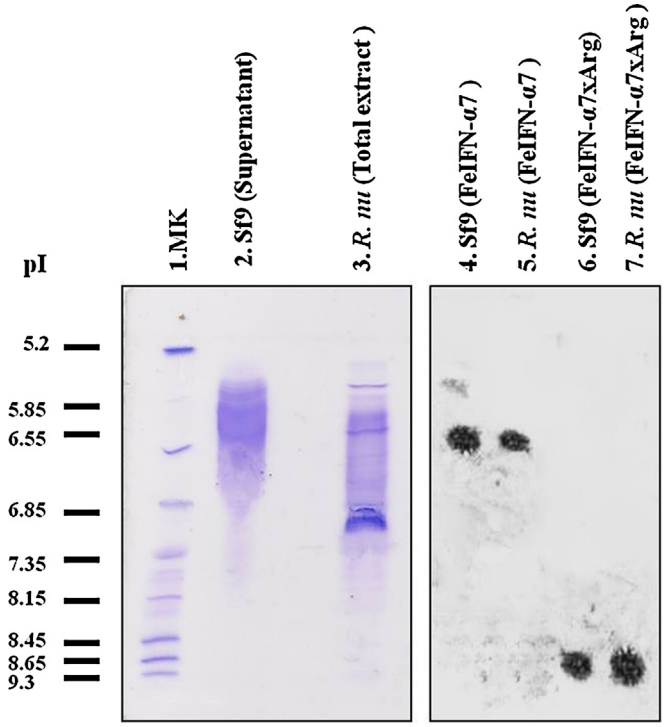

Fig. 5. Isoelectrofocusing (IEF) 3–9 of FeIFN-α7 and FeIFNα7xArg expressed in Sf9 cells and *R. nu* larvae. Lane 1, IEF marker (MK); lane 2, supernatant from Sf9 cell culture at 4 dpi; lane 3, total extract of infected *R. nu* larvae at 2 dpi; lane 4, FeIFN-α7 expressed in Sf9 cells; lane 5, FeIFN-α7 expressed in *R. nu* larvae; lane 6, FeIFN-α7xArg expressed in Sf9 cells; lane 7, FeIFN-α7xArg expressed in *R. nu* larvae. Lanes 1–3 were stained with Coomassie Blue. Lanes 4–7 were developed with DAB after western blot. Western blot was developed with specific polyclonal antiserum raised against a peptide from FeIFN-α7.

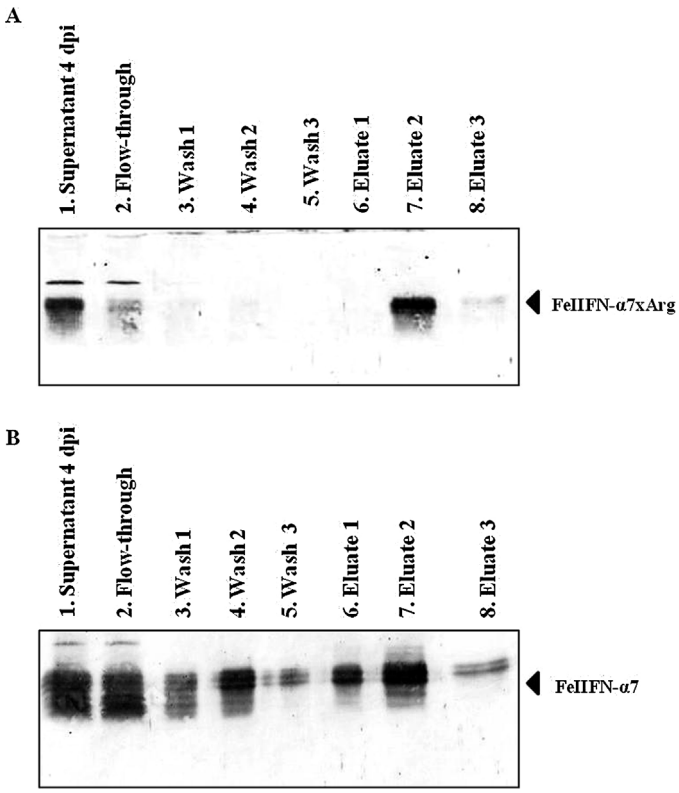

Fig. 6. Analysis by western blot of the purification of FeIFN-α7xArg (A) and FeIFN-α7 (B) expressed in Sf9 cells. Lane 1, Supernatant expression in Sf9 cells (4 dpi); lane 2, ion exchange chromatography (IEC) flow-through; lanes 3–5, IEC washing step with equilibration buffer at different collection times (wash 1–3); lanes 6–8, IEC fraction eluted by 350 mM NaCl in equilibration buffer at different collection times (eluate 1–3). Western blot was developed with specific polyclonal antiserum raised against a peptide from FeIFN-α7.

and C) and *R. nu* larvae (Fig. 8B and D). This is an important feature because the viral inactivation step, frequently by addition of 0.1 N HCl, was not necessary in our strategy. It has been previously described that baculoviral particles do not interact with the cation exchange matrix at a pH above 7.0 [39]. For this reason, the IEC performed at pH 8.0 resulted in a product free of viruses.

3.4. Comparison of biological activity: antiviral and antitumor activity bioassay

The antiviral bioassay indicated that FeIFN-α7 and FeIFN-α7xArg expressed in Sf9 cells and *R. nu* larvae (purified and non-purified) effectively inhibited the infection by VSV. The viability of CRFK cells was increased in all cases up to 100% with 625 U ml^{-1} (Fig. 9A and b). The assay was performed in the concentration range where the interferon was not toxic to the cells [40]. The antitumor bioassay indicated that FeIFN-α7 and FeIFN-α7xArg expressed in Sf9 cells and *R. nu* larvae (purified and non-purified) effectively inhibited the tumor cell proliferation.

Furthermore, we found no difference between FeIFN-α7 and FeIFN-α7xArg and the commercial FeIFN-ω (Virbac) (Fig. 9C and D). Therefore, we demonstrated that the addition of the 8xArg tag at the C-terminus increased the interferon pI from neutral to basic, without affecting the biological activity.

In summary, we analyzed different strategies to produce FeIFN-α7. FeIFN-α7 was expressed efficiently in Sf9 cells and insect larvae. *R. nu* larvae were better hosts for the expression than *S. frugiperda* larvae. In this work, we added the arginine tag directly into the transfer vector. This new transfer vector could be used for increasing the pI of different proteins, including those of low-pI. As we expected, the addition of 8xArg tag residues at the C-terminus of FeIFN-α7 allowed increasing the pI from 6.5 to 9.15. Expression kinetic curves of both FeIFN-α7 and FeIFN-α7xArg were similar, thus indicating that 8xArg tag residues did not affect the biological activity of FeIFN-α7.

In suspension experiments, FeIFN-α7 represented 1.1% of the total protein concentration in the supernatant using low serum media, whereas in whole *R. nu* larvae it represented 0.34% of

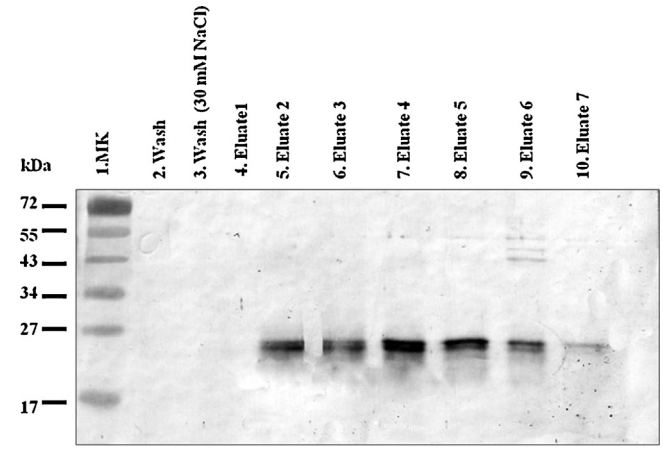

Fig. 7. Analysis by western blot of the purification of FeIFN-α7xArg expressed in *R. nu* larvae. Lane 1, molecular-weight marker (MK); lane 2, IEC washing step (wash) with equilibration buffer; lane 3, IEC washing step with 30 mM NaCl in equilibration buffer; lanes 4–10, fraction eluted by 150 mM NaCl at different collection time (Eluate 1–7). Western blot was developed with specific polyclonal antiserum raised against a peptide from FeIFN-α7.

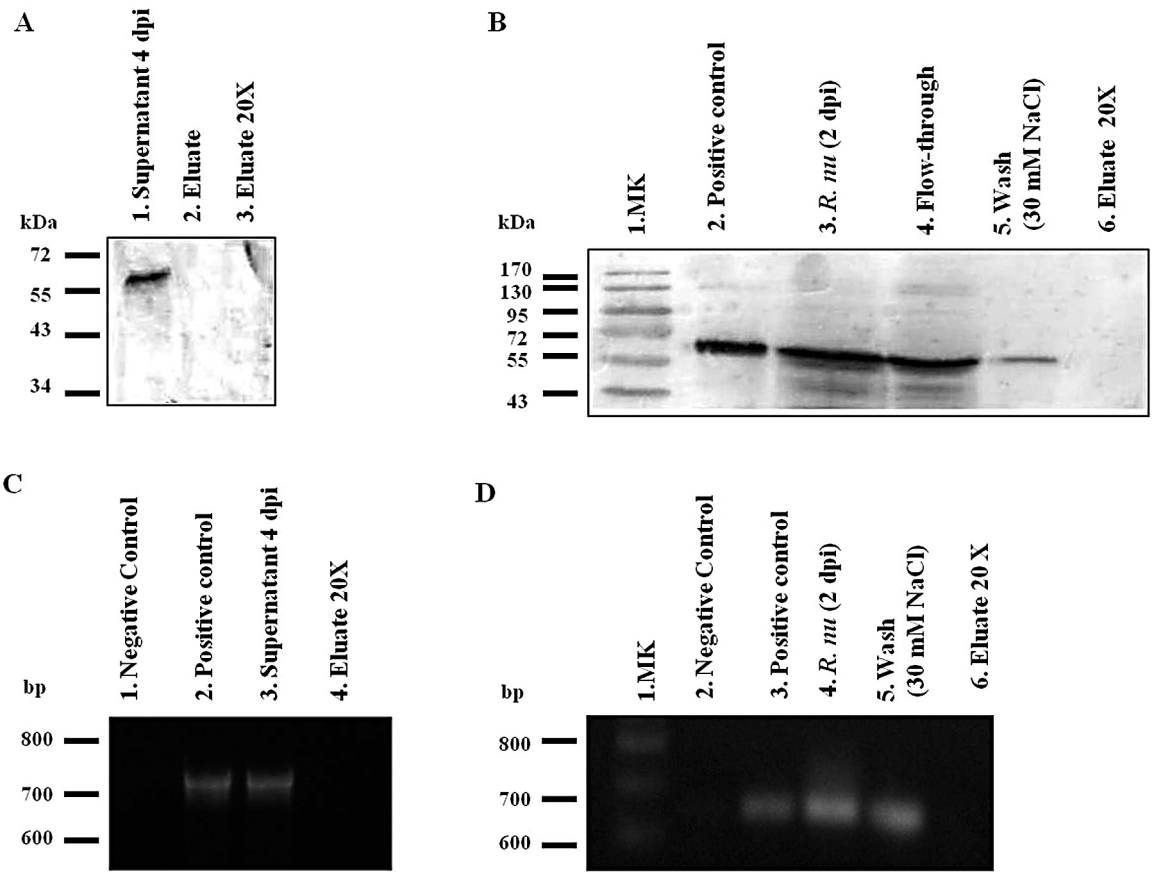

Fig. 8. Virus localization by western blot against GP67 envelope virus protein from the chromatographic fraction of FeIFN-α7xArg expressed in Sf9 cells (A) and *R. nu* larvae (B) and by PCR from the chromatography fraction of FeIFN-α7xArg expressed in Sf9 cells (C) and *R. nu* larvae (D). (A) Lane 1, supernatant from Sf9 cell culture at 4 dpi; lane 2, ion exchange chromatography (IEC) fraction eluted with 350 mM NaCl (eluate); lane 3, IEC fraction (20×) eluted with 350 mM NaCl (eluate 20×). (B) Lane 1, molecular-weight marker (MK); lane 2, positive control (purified virus); lane 3, *R. nu* extract 2 dpi; lane 4, IEC flow-through; lane 5, IEC washing step (wash) with 30 mM NaCl in equilibration buffer; lane 6, IEC fraction eluted (20×) by 150 mM NaCl in equilibration buffer (eluate 20×). (C) Lane 1, negative control; lane 2, positive control (purified virus); lane 3, supernatant from Sf9 cell culture at 4 dpi; lane 4, IEC fraction eluted (20×) by 350 mM NaCl in equilibration buffer (eluate 20×). (D) Lane 1, molecular-weight marker (MK); lane 2, negative control; lane 3, positive control (purified virus); lane 4, larval extract 2 dpi; lane 5, IEC washing step with 30 mM NaCl in equilibration buffer (wash); lane 6, IEC fraction eluted (20×) by 150 mM NaCl in equilibration buffer (eluate). Western blot (A and B) was developed with specific monoclonal antiserum raised against GP67 envelope virus protein. Agarose gel 1% (C and D) was developed after PCR with specific primers.

the total protein. The same amount of recombinant protein was produced in 100 larvae or 300 ml of insect culture, reducing the downstream processing volume three times. To infect 100 larvae and 300 ml of insect cell culture 7×10^7 PFU and 6×10^8 PFU respectively, were needed. Therefore, cell culture required a viral inoculum 8.6 times higher than larvae.

The time of harvest was less in insect larvae (2 dpi) than in insect culture (4 dpi). In addition, the cell culture needed to be expanded before FeIFN-α7xArg production. The whole process takes 10 days and needs 85.5% more virus titer than the optimized larval method. After the viral stock is ready, the complete process using larvae, including expression and purification, takes only 3 days. Particularly, the use of insect larvae is interesting because neither large quantities of culture medium nor sophisticated instrumentation are required. Protein production processes based on insect larvae also enclose several drawbacks. For example, larva must be injected one by one. However, it is possible to construct a polh+ virus for oral infection. This is a simpler methodology chosen when a large number of larvae have to be inoculated [23]. In a previously work, we have proved that the performance of polh+ and polh− as horseradish peroxidase (HRP) expression vectors was the same either in insect cell cultures or in larvae, thus indicating that there was not interference between polyhedrin and HRP expression [22]. Trials should be performed to determine the influence of polyhedrin in the expression of interferon. Another problem related to the production of recombinant protein in larvae is the melanization process that occurs during the homogenization step. This process may be controlled to avoid large aggregates of proteins that seriously complicate the purification of the recombinant protein [27]. In order to prevent this, reduced glutathione was included in the extraction buffer. Finally, the purification process of recombinant proteins from larvae is more difficult than in cells by the presence of different tissues and the presence of a lot of non-characterized contaminating proteins and lipids. On the other hand, the baculovirus-insect system is an excellent eukaryotic expression system for the production of proteins for biotechnological applications [18]. Even when processes based on insect cells are gaining ground rapidly among expression systems, the commercial production of recombinant proteins is limited because of their high cost. A large investment in highly technical equipment and raw materials is required and there are several problems associated with the scaling up that negatively affect the protein production [41]. Therefore, large-scale production using insect larvae as expression hosts is becoming a promising strategy as the manufacturing cost could be reduced in comparison with cell cultures [17,42]. The harvest time for recombinant protein expression in insect larvae should be monitored in each case because it may vary with each particular protein. It is important to inject a small number of insect larvae with recombinant baculovirus as a preliminary step in order to verify the protein expression in whole insects and select the

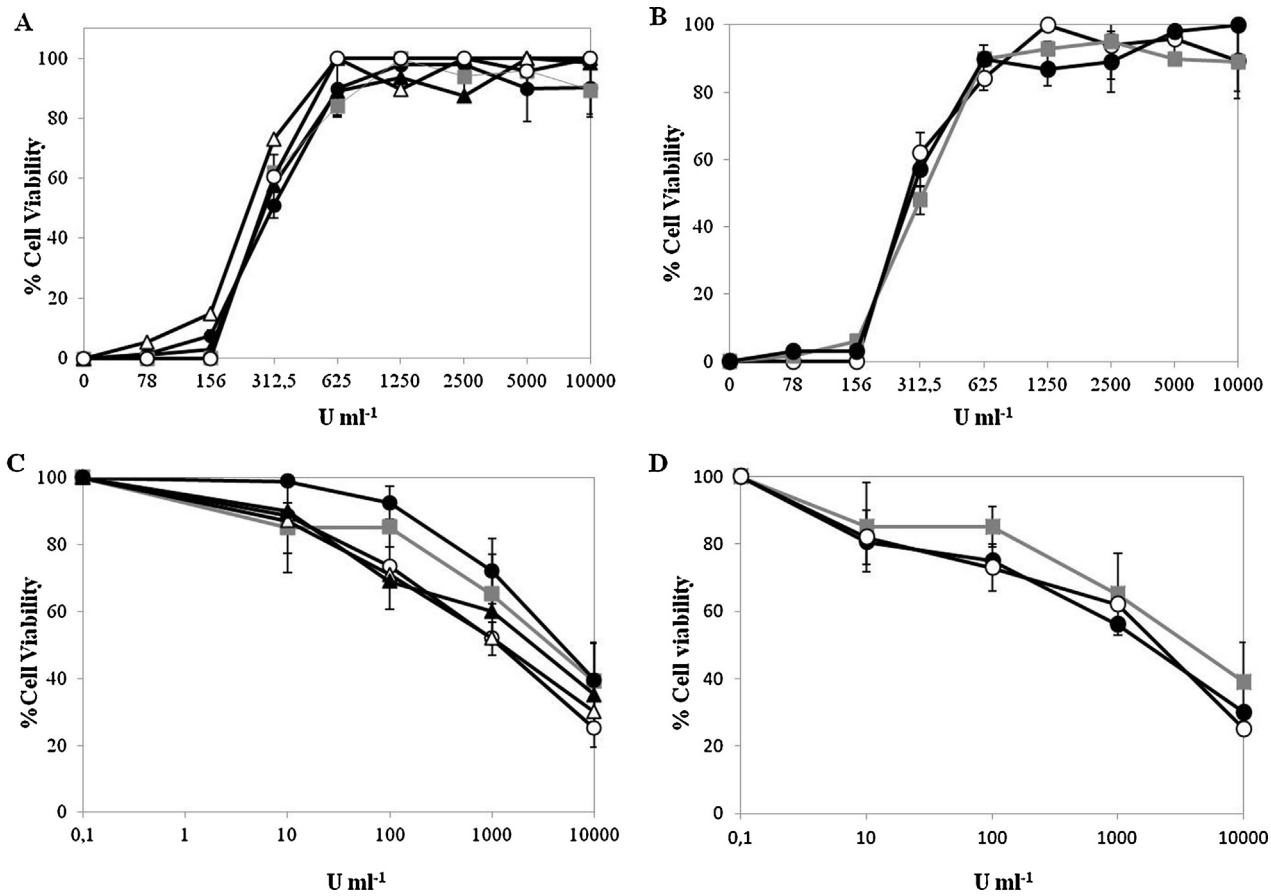

Fig. 9. Antiviral and antitumor activity assay. (A) % Viability of CRFK cells infected with vesicular stomatitis virus (VSV) and treated with increasing concentrations of recombinant FeIFN-α7 or FeIFN-α7xArg expressed in Sf9 cells or *R. nu* larvae or standard FeIFN-ω. (B) % Viability of CRFK cells infected with vesicular stomatitis virus (VSV) and treated with increasing concentrations of purified recombinant FeIFN-α7xArg expressed in Sf9 cells or *R. nu* larvae or standard FeIFN-ω. (C) % Viability of feline mammary carcinoma cells treated with increasing concentrations of recombinant FeIFN-α7 or FeIFN-α7xArg and expressed in Sf9 cells or *R. nu* larvae or standard FeIFN-ω. (D) % Viability of feline mammary carcinoma cells treated with increasing concentrations of purified recombinant FeIFN-α7xArg and expressed in Sf9 cells or *R. nu* larvae or standard FeIFN-ω. (▲) FeIFN-α7 expressed in Sf9 cells, (△) FeIFN-α7 expressed in *R. nu* larvae, (●) FeIFN-α7xArg expressed in Sf9 cells, (○) FeIFN-α7xArg expressed in *R. nu* larvae, (■) FeIFN-ω (Virbac). Results are expressed as the average ± standard deviation ($n = 3$).

optimal day for protein harvesting. Once this step has been carried out and the protein quantified, the process can be linearly scaled up. The availability of automated rearing equipment and the fact that the larvae are nonallergenic to human handlers make scale-up and mass production of recombinant proteins very attractive for commercial protein production. With an automated facility for mass rearing and controlled conditions it is possible to scale up to kilograms of protein-containing larvae per week [20].

In contrast with *B. mori* larvae, *R. nu* larvae are small and, as a consequence, the recombinant protein must be recovered from the larval extract where the initial protein contamination is higher than in hemolymph. Even when the recombinant protein is secreted in hemolymph of *R. nu* larvae, it is more convenient to obtain an extract by simple homogenization. For this reason, the strategy of downstream processing is very important in this case and IEC is interesting because it preserved FeIFN-α7xArg with biological activity and free of viral particles in only one step. The need for additional purification steps should be analyzed for veterinary applications. As an example, hemolymph from *B. mori* larvae containing antigens or virus-like particles can be directly used as a veterinary vaccine formulation without purification because silkworm hemolymph does not cause any anaphylactic reaction in immunized animals [43]. The IEC is a cost–effective downstream processing method [44]. The strategy to increase the pI of FeIFN-α7 was useful to purify FeIFN-α7 from Sf9 cell cultures and total larval extract at pH 8.0 by IEC. This strategy could be useful in different hosts for the selective adsorption of recombinant proteins on an ion exchange matrix. The purification performance will depend on the difference between the pI of the contaminant proteins from the particular host and the pI of the recombinant protein after the addition of the arginine tag.

4. Conclusions

R. nu larvae, a plague without any economic value, appeared as an interesting host to express, with high yield, biologically active FeIFN-α7xArg. Purification of FeIFN-α7xArg by IEC directly from the larval extract was a low-cost strategy easy for scaling up. While FeIFN-α7xArg and FeIFN-ω had identical biological activity, the main achievement of this work was the profitable production method.

Acknowledgements

GCG, LMEF, MSV, OT, OC, MVM are career researchers of the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET). AMT is a research fellow of the CONICET.

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica de la República Argentina [PICT 0606; PICT 2008-1006; PICT Bicentenario 2010-0610],

Universidad de Buenos Aires, CONICET [PIP 00052] and Ministerio de Salud Pública de la República Argentina.

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