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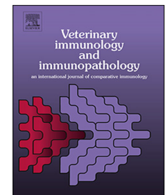
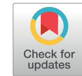


Enhancing purification and plasma stability of porcine interferon-α/γ by fusion to elastin-like polypeptide



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ABSTRACT

The clinical use of recombinant interferons (rIFNs) is limited by higher purification cost and quick clearance from circulation. Elastin-like polypeptides (ELPs) are a novel tag for recombinant protein purification and half-life extension. In this study, we evaluated the feasibility of ELP fusion for simple purification and half-life extension of recombinant porcine IFNs (rPoIFNs). After construction of five different fusion expression vectors, we optimized the conditions for soluble protein expression and purification. SDS-PAGE analysis showed that, unlike PoIFNα-His and PoIFNγ-His, PoIFNα-ELP, ELP-PoIFNα and PoIFNαγ-ELP were expressed mainly as soluble proteins at 20 °C. The optimal conditions for the inverse transition cycling (ITC) of three ELP fusion proteins were 2 M NaCl at 28 °C. After two rounds of ITC, the three ELP fusion proteins were purified to more than 90% purities, which were comparable to that of affinity-purified PoIFNα-His and PoIFNγ-His. Cytopathic effect inhibition assay showed that the five rPoIFNs had potent but different antiviral activities against two different viruses on two different cell types. The plasma solubility assay showed that the three ELP-fused rPoIFNs remained as soluble proteins under the physical conditions. The plasma stability of three ELP-fused rPoIFNs was significantly improved in comparison with that of PoIFN-α. These data suggest that ELP fusion is a feasible strategy to enhance purification and plasma stability of rPoIFNs.

1. Introduction

Interferons (IFNs) are a group of cytokines with antiviral, immunomodulatory and antiproliferative effects (Chelbi-Alix and Wietzerbin, 2007). Recombinant porcine IFNs (rPoIFNs), mainly rPoIFN-α and rPoIFN-γ, have been expressed in *E. coli* (Lefèvre et al., 1990), yeasts (Wang et al., 2002), insect cells (Vandenbroeck et al., 1994) or mammalian cells (Cencic et al., 1999) since their first gene cloning. Although they are highly active against a variety of pig viruses (Xia et al., 2005), these rPoIFNs are expressed with affinity tags and thus require expensive chromatographic columns for purification (Zhao et al., 2007). More importantly, the clinical efficacy of unmodified rPoIFNs may be limited due to their small molecular size and quick clearance from circulation (Walls and Loughran, 2011).

Polyethylene glycol modification or pegylation is a well-established strategy for protein half-life extension, and the two versions of pegylated human IFN-α have been used clinically to treat chronic hepatitis (Zeuzem et al., 2000; Heathcote et al., 2000). However, pegylation has the drawbacks of low efficiency, poor clinical efficacy and significant side-effects (Bota et al., 2013). Human serum albumin (HSA) fusion is an alternative strategy for half-life extension of human IFN-α with higher efficiency and less side-effects (Osborn et al., 2002; Zeuzem, 2008). However, recent clinical trials did not show the superior efficacy of IFNα-HSA in patients. In addition, the low yield and high cost of HSA fusion proteins produced in eukaryotic cells significantly limit their clinical use (Hu et al., 2015).

Elastin-like polypeptides (ELPs) are genetically synthetic biopolymers composed of Val-Pro-Gly-Xaa-Gly repeats, where the guest residue Xaa can be any amino acid except proline (Meyer and Chilkoti, 2002). These polymers can undergo reversible phase transition from soluble forms into aggregates as temperature increases (Urry, 1992). This unique property, together with the excellent biocompatibility and low immunogenicity, makes ELPs very useful for a wide variety of biomedical applications, including protein purification and drug delivery (Mackay and Chilkoti, 2008). More recently, ELP fusion has been used to enhance the pharmacokinetics and anti-tumor efficacy of human IFN-α with high yield, low cost and well-retained bioactivity (Hu et al., 2015). Therefore, the main objective of this study was to broaden the use of ELP fusion technology for simple purification and half-life extension of PoIFNs.

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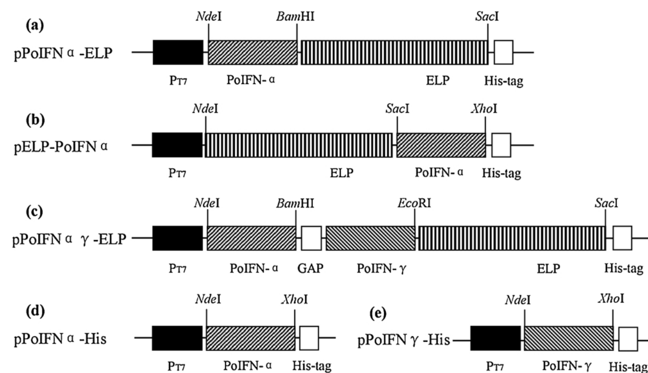

Fig. 1. The schematic structures of expression vectors used in this study. PT7 indicates T7 promoter. The restriction sites used for gene cloning and coding sequences for PoIFN-α, PoIFN-γ, ELP, His tag and GAP linker are indicated.

2. Materials and methods

2.1. Vector construction

ELP fusion expression vector pET-ELP was constructed by cloning the coding sequence for 110 repeats of VPGVG block (Banki et al., 2005) into pET-30a (+) vector (Liu et al., 2014). The coding sequences for the mature polypeptides of PoIFN-α6 (GenBank: AY345969; Sang et al., 2014) and PoIFN-γ (GenBank: AY188090) were adapted to *E. coli* codon usage using Java Codon Adaption Tool (Grote et al., 2005) to facilitate their expression in *E. coli*. To compare ELP fusion order on protein expression and/or antiviral activity, two different fusion expression vectors, namely pPoIFNα-ELP and pELP-PoIFNα (Fig. 1a and 1b), were constructed by cloning PoIFN-α coding sequence upstream or downstream of ELP coding sequence in the pET-ELP vector. To enhance PoIFN bioactivities, the fusion expression vector pPoIFNαγ-ELP (Fig. 1c) was constructed by fusing PoIFN-γ coding sequence at the *C*-terminus of PoIFN-α coding sequence in the pPoIFNα-ELP vector. For the comparison purpose, two His-tagged expression vectors, namely pET-PoIFNα (Fig. 1d) and pET-IFNγ (Fig. 1e), were constructed by cloning the PoIFN-α or PoIFN-γ coding sequences into pET-30a (+) vector. The expressed proteins of five recombinant vectors were called PoIFNα-ELP, ELP-PoIFNα, PoIFNαγ-ELP, PoIFNα-His and PoIFNγ-His, respectively.

2.2. Fusion protein expression

The five recombinant vectors were transformed individually into *E. coli* strain BL21 (DE3), and 10 mL of Luria broth cultures containing kanamycin (50 μg/mL) were grown overnight at 37 °C in an orbital shaker. These cultures were diluted (1:100) in 1 L of 2 × YT medium (10 *g* yeast extract, 16 *g* tryptone, 5 *g* NaCl/L) containing the same antibiotic. After 6-h growth at 37 °C, the expression of fusion proteins was induced first with 0.2 mM IPTG (final concentration) for 6 h at 37 °C. To optimize the conditions for soluble protein expression, the protein expression was induced with different concentrations of IPTG at different temperatures. After sonication treatment and centrifugation at 12,000 *g*, both supernatants and pellets were separated on 12% SDS-PAGE gels for protein expression and solubility analysis.

2.3. Fusion protein purification

After IPTG induction, the cells were harvested by 10-min centrifugation at 5000 *g*, suspended in 50 mL of PBS (pH 7.2), and disrupted three times at 800 bar at 4 °C using High Pressure Cell Disruptor (JNBIO, Guangzhou, China). After 20-min centrifugation at 12,000 *g*, the samples were collected for protein purification. The inverse transition cycling (ITC) for ELP fusion protein purification was performed as described previously (Wu et al., 2016). Briefly, each cell lysate (200 μL) was incubated first with 2 M NaCl (final concentration) for 10 min at different temperatures, and then with different concentrations of NaCl at the optimized temperature. After 5-min centrifugation at 12,000 *g* at room temperature (hot spin), ELP-protein pellet was collected, suspended in cold PBS, and incubated on ice until complete dissolution. After 10-min centrifugation at 12,000 *g* at 4 °C (cold spin), the soluble protein was collected for another round of ITC. The protein samples before and after ITC were analyzed by 12% SDS–PAGE for protein recovery and purity using Molecular Imager® Gel Doc™ XR + System with Image Lab™ Software (BIO-RAD). Finally, the three ELP fusion proteins were purified from 1 L of bacterial cultures by ITC under the optimized conditions. The control proteins PoIFNα-His and PoIFNγ-His were purified under denatured conditions using His-Tagged Protein Purification Kit (CWBIO, Beijing, China) by following the manufacturer's instruction. All of the purified proteins were dissolved in 8 M urea, dialyzed subsequently against 6, 4, 2 M urea, and PBS (pH 7.2).

2.4. Western blotting

The five rPoIFNs were identified by Western blotting using His-specific antibody since the presence of a His tag at their *C*-termini. Briefly, each protein sample (1 μg) was separated on 12% SDS-PAGE gel and electro-transferred onto nitrocellulose membrane. After blocking with 5% skim milk powder in PBS for 2 h at 37 °C, the membrane was incubated first with 1: 4000 mouse anti-His antibody (Sangon Biotech, China) and then with 1: 10,000 DyLight 800-labeled goat anti-mouse IgG (Rockland, USA) for 1 h at 37 °C. After washing in PBS, the hybridization signal was scanned using Odyssey Infrared Imaging System (LI − COR Biosciences, USA).

2.5. Antiviral assay

The antiviral activities of five rPoIFNs against vesicular stomatitis virus (VSV) and pseudorabies virus (PRV) were measured by cytopathic effect (CPE) inhibition assay as previously described (Osborn et al., 2002) using ELP-fused C2 domain of streptococcal G protein (Xia et al., 2017) as the negative control. Briefly, MDBK or PK-15 cells were seeded on 96-well plates and grown to 90% confluence. Different rPoIFNs (1 mg/mL) were serially diluted with PBS (pH 7.2) and added in quadruplicates to the wells (100 μL). After 24-h incubation at 37 °C, optimal concentrations (100 $TCID_{50}$) of the viruses were added. After incubation for additional 24 h, the cell viabilities were measured at 580 nm on an ELISA reader after crystal violet staining. The antiviral activities (IU/mg) of rPoIFNs were calculated using the standard curve generated with swine leukocyte IFN-α (PoIFN-α) (Sichuan Red Biological Technology Co., Ltd, China). The standard deviation was calculated using Microsoft Office Excel 2003.

2.6. Plasma solubility assay

The five rPoIFNs were mixed individually with 50% rat plasma at 10 μg/mL and incubated for 30 min at 37 °C. After 20-min centrifugation at 12,000 *g* to remove protein aggregates, the supernatants were collected for anti-VSV activity assay on MDBK cells as described.

2.7. Plasma stability assay

The plasma stabilities of five rPoIFNs were measured by CPE inhibition assay as described (Osborn et al., 2002) using PoIFN-α (2.6×10^4 IU/mg) as the control. Briefly, rPoIFN or PoIFN-α was mixed with 50% pig plasma at 10 μg/mL and incubated for 0.5, 2, 4, 8, 16 or 32 at 37 °C. After 20-min centrifugation at 12,000 *g* to remove debris, the supernatant was collected and the remaining anti-VSV activity was assayed on MDBK cells as described.

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