Protein Expression and Purification 153 (2019) 53–58

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Half-life extension of porcine interferon-α by fusion to the IgG-binding domain of streptococcal G protein

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ARTICLE INFO

Keywords:
Porcine interferon-α
Streptococcal protein G domain C2
Half-life extension

ABSTRACT

Recombinant interferon-α (rIFN-α) has been widely used for treating viral infections. However, the clinical efficacy of unmodified rIFN-α is limited due to small molecular size and rapid clearance from circulation. In this study we developed a novel strategy for half-life extension of porcine IFN-α (PoIFN-α) by fusion to the immunoglobulin (Ig)-binding C2 domain of streptococcal protein G (SPG). The coding sequences for PoIFN-α6 and SPG C2 domain, with a tobacco etch virus (TEV) protease recognition sequence introduced at the 5-end, were cloned into an elastin-like polypeptide (ELP) fusion expression vector and expressed as an ELP-PoIFNα-C2 fusion protein. After optimization of the conditions for soluble protein expression and purification, the fusion protein was purified to more than 90% purity by two rounds of inverse transition cycling (ITC) in the presence of 0.5% Triton X-100. After cleavage with self-aggregating peptide ELK-16-tagged tobacco etch virus protease, the protease was removed by quick centrifugation and PoIFNα-C2 protein was recovered by an additional round of ITC with 98% purity. Western blotting analysis showed that PoIFNα-C2 protein had the specific affinity for pig IgG binding. The antiviral assay showed that PoIFNα-C2 protein had potent antiviral activities against vesicular stomatitis virus and porcine pseudorabies virus. After single intravenous or subcutaneous injection into rats, PoIFNα-C2 protein showed 16- or 4-fold increase in serum half-life with significantly improved bioavailability.

1. Introduction

Interferons (IFNs) are a group of cytokines with antiviral and immune regulatory effects [1]. In mammals, IFNs are diversified into three different types. Type I IFNs, particularly IFN-α and IFN-β, are among the most widely used recombinant proteins for treating human cancers and viral infections [2]. Recombinant porcine IFN-α (rPoIFN-α) has been expressed in *E. coli* with high activity against a variety of viruses [3,4]. Like other rIFNs, however, the clinical efficacy of unmodified rPoIFN-α may be limited due to small molecular size and rapid clearance from circulation [5].

Polyethylene glycol modification is a well-established strategy for protein half-life extension, and pegylated human IFN-α formulations have been proved for clinical use [6,7]. However, the pegylated IFN-α has drawbacks of poor efficacy and significant adverse effects [8]. Human serum albumin (HSA) fusion is the alternative technology for half-life extension of human IFN-α with higher efficacy and less adverse effects [9]. Recently, albumin-binding domain antibody fusion has also been used to extend the serum half-life of human IFN-α [10]. However, these rIFNs are expressed with an affinity tag, and thus require expensive columns, proteases and additional chromatography steps for purification [11].

Elastin-like polypeptides (ELPs) are synthetic biopolymers composed of pentapeptide repeats of VPGXG, where the guest residue X can be any amino acid except proline [12]. These polymers undergo reversible phase transition from soluble forms into aggregates as the temperature increase [13]. Significantly, ELP fusion proteins retain the temperature-sensitive phase transition property, allowing easy purification by inverse transition cycling (ITC) [14].

Immunoglobulin (Ig)-binding proteins, mainly staphylococcal protein A (SPA) and streptococcal protein G (SPG), are commonly used as the ligand choice for antibody purification. More recently, the Ig-binding domains of SPA and SPG have been used to extend the serum half-life of small recombinant antibodies [15]. In addition, our recent study showed that ELP-fused SPG C2 domain can bind to mammalian IgGs and chicken IgY [16].

In this study we developed a novel strategy for serum half-life extension of PoIFN-α by fusing to SPG C2 domain. The PoIFNα-C2 fusion protein was first expressed with an ELP tag and purified by ITC. After removing the ELP tag with tobacco etch virus (TEV) protease, we

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https://doi.org/10.1016/j.pep.2018.08.012
Received 9 June 2018; Received in revised form 23 August 2018; Accepted 23 August 2018
Available online 27 August 2018

compared the antiviral activities and pharmacokinetics of PoIFNα-C2 protein with PoIFN-α standard.

2. Materials and methods

2.1. Plasmid, cells and viruses

The pET/EI-GFP vector containing 110 repeats of GVGVP block [17] was kindly provided Professor David Wood at the Princeton University, USA. The ELP cloning sequence is subcloned into pET-30a (+) vector as an *Nde*I/*Sac*I segment and the modified vector is called pET-ELP [18]. Bovine kidney cell line MDBK and pig kidney cell line PK-15 (ATCC, USA) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg ml^{-1}). Vesicular stomatitis virus (VSV) New Jersey strain and porcine pseudorabies virus (PRV) Bartha K61 strain (ATCC, USA) were propagated and titrated on MDBK cells.

2.2. Vector construction

The coding sequences for the mature polypeptide of PoIFN-α6 (GenBank accession no. AY345969) and SPG C2 domain (GenBank accession no. X06173.1) were adapted to *E. coli* codon usage using Java Codon Adaption Tool [19]. The synthetic sequence for PoIFN-α6, with a TEV protease recognition sequence at the 5′ end, was cloned into pET-ELP vector as a *Sal*I/*Bam*HI segment. The synthetic sequence for SPG C2 domain was cloned downstream of PoIFN-α6 sequence as a *Bam*HI/*Xho*I segment. The recombinant vector was called pELP-PoIFNα-C2 (Fig. 1A), and the amino acid sequence of ELP-PoIFNα-C2 fusion protein is presented as a data in brief file.

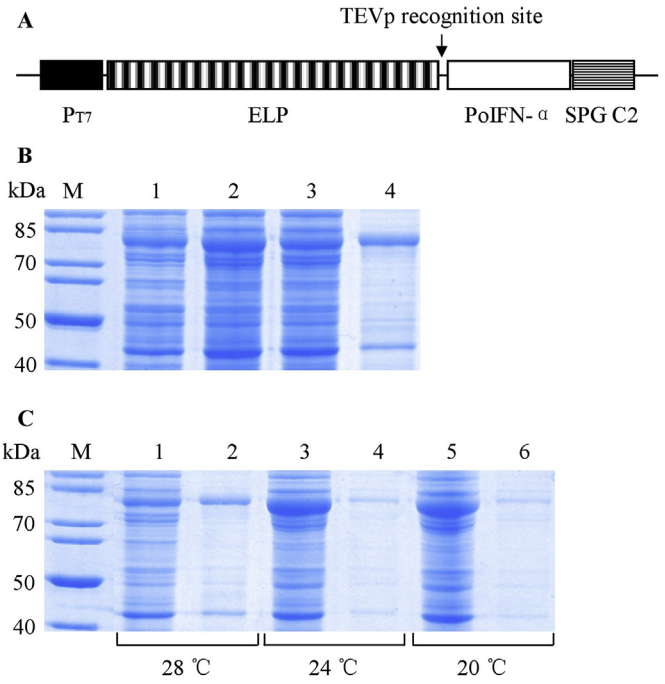

Fig. 1. Expression of ELP-PoIFNα-C2 fusion protein in *E. coli*. **A**. The schematic structure of fusion expression vector. PT7 indicates T7 promoter. **B**. Analysis of fusion protein expression and solubility. The protein expression was induced with 0.2 mM IPTG for 6 h at 37 °C. The cell lysate before IPTG induction (1), after IPTG induction (2), and the supernatant (3) and pellet (4) after centrifugation were analyzed by 12% SDS-PAGE. **C**. Optimization of the temperature for soluble protein expression. The protein expression was induced with 0.2 mM IPTG for 24 h at indicated temperatures. After 10-min centrifugation at 12,000 g, both soluble (1, 3, 5) and insoluble (2, 4, 6) fractions of centrifuged cell lysate were analyzed by 12% SDS-PAGE.

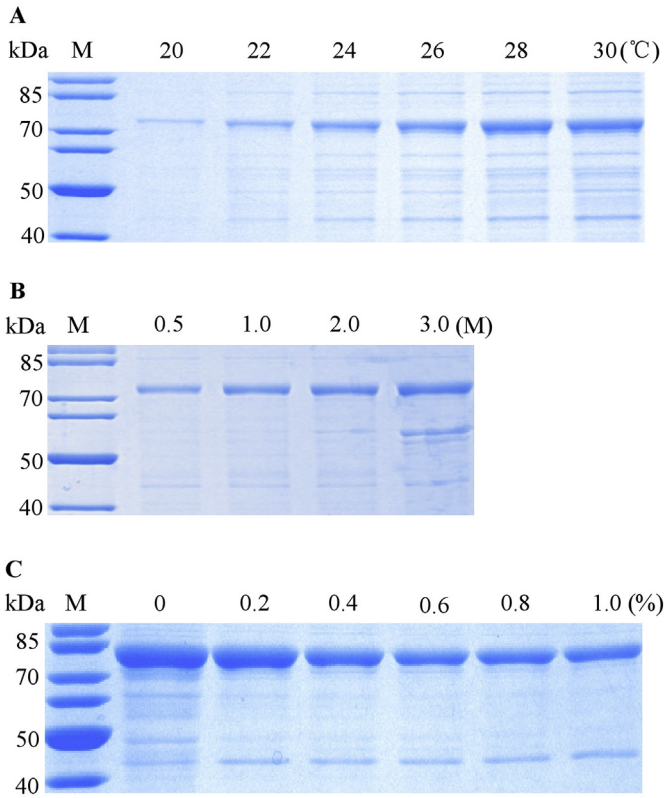

Fig. 2. Modification of ITC conditions for ELP-PoIFNα-C2 protein purification. **A**. Optimization of temperature for phase transition. The ITC was performed with 3 M NaCl for 10 min at indicated temperatures. **B**. Optimization of salt concentration for phase transition. The ITC was performed with different concentrations of NaCl at 28 °C. **C**. Optimization of Triton X-100 concentration for protein purification. The ITC was performed with 2 M NaCl at 28 °C in the presence of indicated Triton X-100 concentrations. The samples were analyzed by 12% SDS-PAGE.

2.3. Fusion protein expression

The pELP-PoIFNα-C2 vector was transformed into *E. coli* strain BLR (DE3), and 5 mL of Luria broth culture supplemented with 50 μg mL^{-1} kanamycin was grown overnight at 37 °C in an orbital shaker. The culture was 1:100 diluted in 2 × YT medium (10 g L^{-1} yeast extract, 16 g L^{-1} tryptone, 5 g L^{-1} NaCl) containing the same antibiotic, and grown for 6 h at 37 °C. To detect the temperature for soluble protein expression, the cell culture was induced with 0.2 mM IPTG for 24 h at different temperatures.

2.4. Fusion protein purification

After IPTG induction, the cells were harvested by 10-min centrifugation at 5000 g, suspended in PBS (pH7.2), and disrupted two times at 1300 bar using High Pressure Cell Disruptor (JNBIO, China). After 10-min centrifugation at 12,000 g, the supernatant was collected for protein purification. The ITC for ELP-PoIFNα-C2 protein purification was performed as previously described [20] with slight modification. Briefly, the clarified bacterial extract was incubated with 0.5% polyethyleneimine for 15 min at 4 °C to precipitate bacterial genomic DNA. After 10-min centrifugation at 12,000 g, the supernatant was collected for ITC. First, the ITC was performed with 3 M NaCl for 10 min at different temperatures or with different concentrations of NaCl at the optimized temperature. Then, the ITC was performed in the presence of different concentrations Triton X-100. Finally, the ITC was performed with optimized concentration of NaCl at optimized temperature in the presence of optimized Triton X-100 concentration. After

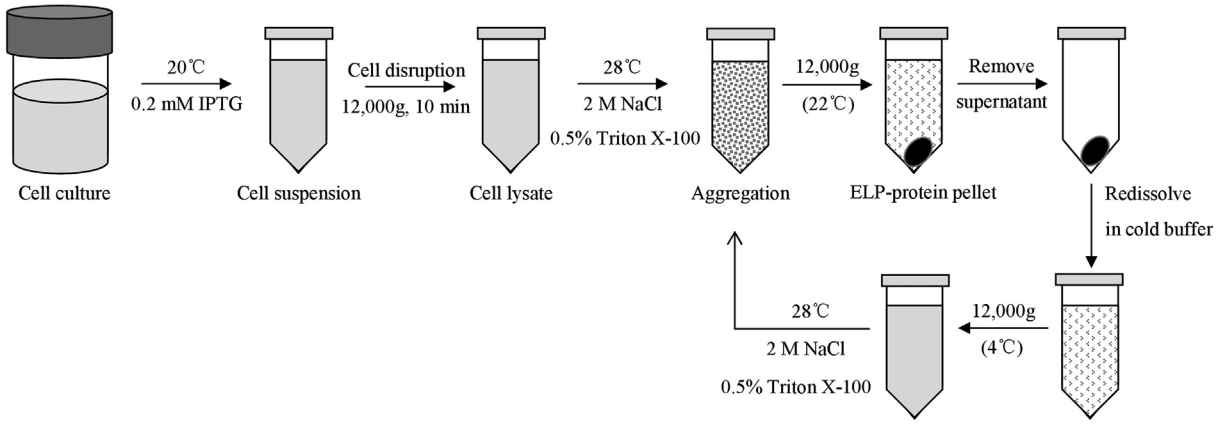

Fig. 3. Summarized ITC protocol for ELP-PoIFNα-C2 protein purification.

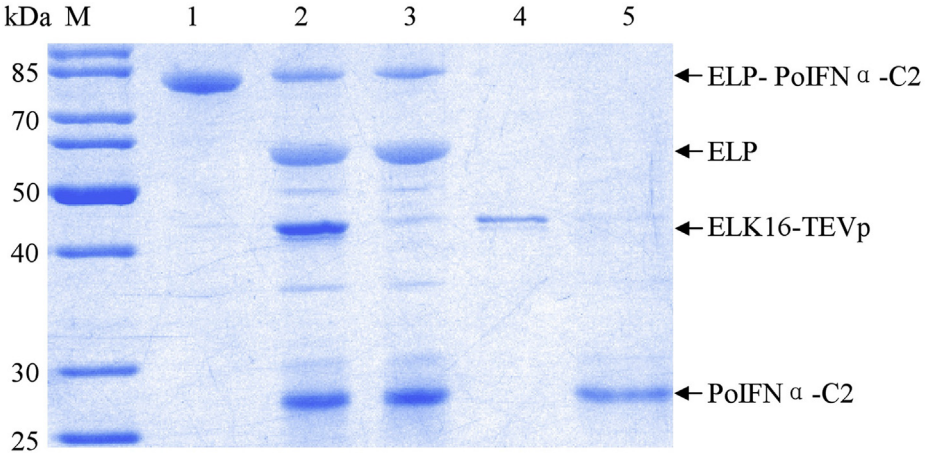

Fig. 4. ELP tag cleavage and PoIFNα-C2 protein recovery. The purified ELP-PoIFNα-C2 protein (1), TEVp digest (2), supernatant (3) and pellet (4) after centrifugation, and PoIFNα-C2 protein recovered by ITC (5) were analyzed by 12% SDS-PAGE.

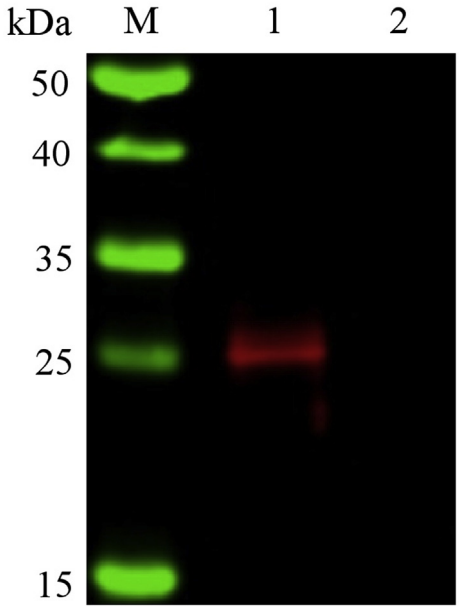

Fig. 5. Western blotting analysis of PoIFNα-C2 protein binding to pig IgG. PoIFNα-C2 protein (1) and PoIFN-α standard (2) were transferred onto nitrocellulose membrane and detected using fluorescently labeled pig IgG.

5-min centrifugation at 12,000 g at room temperature, the fusion protein was collected and suspended in cold PBS (pH 7.2). After 60-min incubation on ice, the sample was centrifuged for 10 min at 12,000 g, and the supernatant was collected for another round of ITC under the same conditions.

2.5. Tag cleavage and target protein recovery

The ELP tag was cleaved off ELP-PoIFNα-C2 fusion using self-aggregating peptide ELK16-tagged TEV protease (ELK16-TEVp) as previously described [21]. Briefly, the purified fusion protein was suspended in the protease cleavage buffer and incubated at 4 °C to complete dissolution. After 10-min centrifugation at 14,000 g to remove insoluble debris, the ELK16-TEVp was added and incubated for 6 h at 30 °C. After 5-min centrifugation at 1000 g to remove the protease, the PoIFNα-C2 protein was recovered by an additional round of ITC as described. The protein samples before and after ITC were analyzed by 12% SDS-PAGE for purity using Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software (BIO-RAD, USA) after Commassie blue staining.

2.6. Western blotting

Both PoIFNα-C2 protein and swine leukocyte IFN-α (Sichuan Shihong Biotechnology, China) were separated by 12% SDS-PAGE, and transferred onto nitrocellulose membrane. After blocking with 5% skim milk powder and 0.1% Tween 20 in phosphate-buffered saline (PBST), the membrane was incubated with 1:10000 DyLight 800-labeled pig

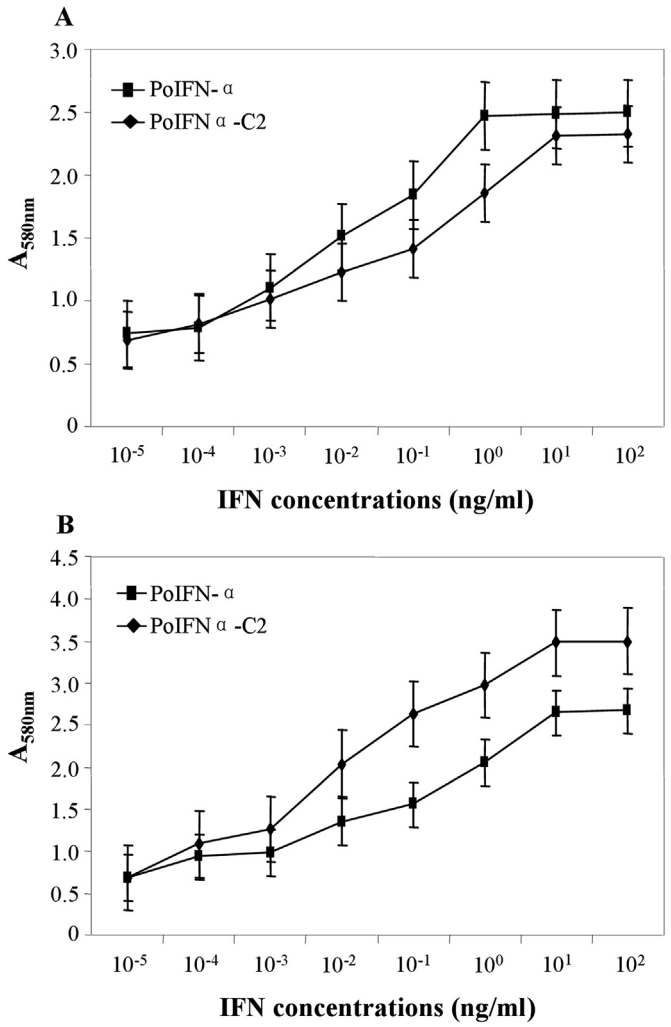

Fig. 6. Comparison of antiviral activities between PoIFNα-C2 protein and PoIFN-α standard. **A**. Anti-VSV activities on MBDK cells. **B**. Anti-PRV activities on PK-15 cells.

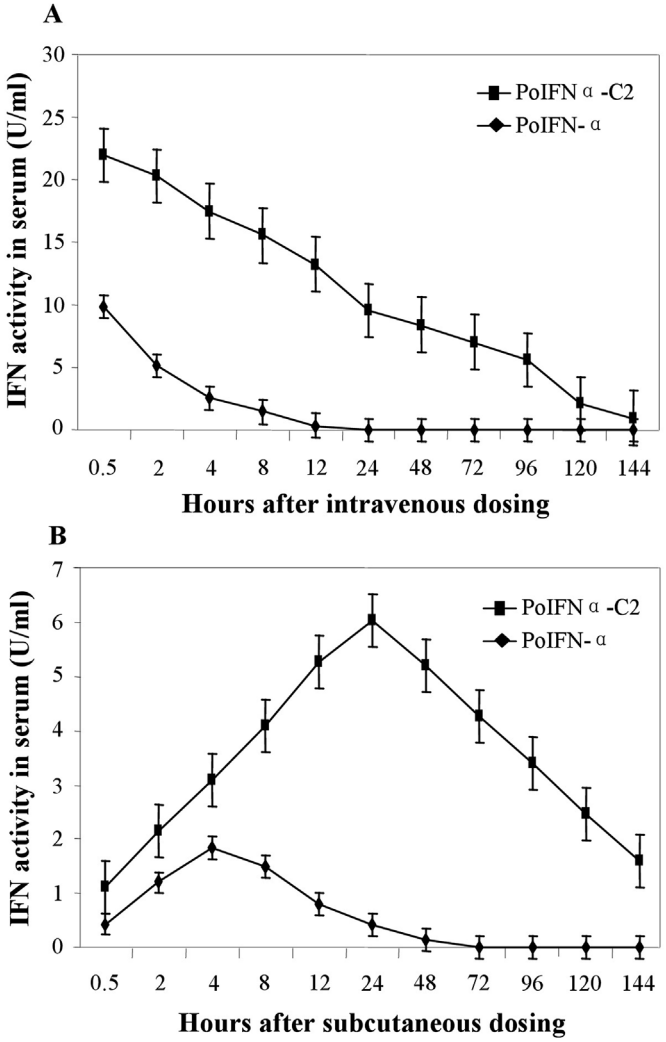

Fig. 7. Comparison of pharmacokinetics between PoIFNα-C2 protein and PoIFN-α standard. **A**. Intravenous injection. **B**. Subcutaneous injection. The remaining anti-VSV activities in rat serum samples were assayed on MDBK cells.

IgG (Rockland, USA) for 1 h at room temperature, and the hybridization signal was scanned using Odyssey Infrared Imaging System (LI-COR Biosciences, USA) after washing with PBST.

2.7. Antiviral activity assay

The anti-VSV and anti-PRV activities of PoIFNα-C2 protein were compared to that of PoIFN-α standard using cytopathic effect (CPE) inhibition assay on MDBK or PK-15 cells as previously described [10]. Briefly, 90% confluent cells on 96-well plates were treated in triplicates with different concentrations of PoIFNα-C2 protein or PoIFN-α standard. After incubation for 24 h at 37 °C, optimal concentrations (100 $TCID_{50}$) of viruses were added. After incubation for additional 24 h, the cells were stained with 1% crystal violet in 15% ethanol, and extracted with 70% ethanol and 1% acetic acid. After absorbance determination at 580 nm on an ELISA reader, the antiviral activities (IU/μM protein) were calculated according to the standard curve generated using swine leukocyte IFN-α.

2.8. Pharmacokinetic analysis

The pharmacokinetics of PoIFNα-C2 protein was compared to that of PoIFN-α standard as previously described [10]. Briefly, twelve male Sprague Dawley rats weighing 180–220 g were randomly assigned to four groups. Each group was subcutaneously or intravenously injected with a single dose (2.5 mg kg^{-1}) of PoIFNα-C2 protein or PoIFN-α standard. The serum samples were collected at 0.5, 2.0, 4.0, 8.0, 12, 24, 48, 72, 96, 120 and 144 h post injection, and assayed for anti-VSV activity as described.

3. Results and discussion

Affinity tags are commonly used to facilitate recombinant protein purification. However, the fusion tag usually needs to be removed from the final product, which requires expensive proteases and additional chromatography steps [11]. More importantly, the clinical efficacy of unmodified rIFNs is limited due to quick clearance from circulation [5]. In this study, we developed a novel strategy for half-life extension of PoIFN-α by fusing to SPG C2 domain.

3.1. Expression of ELP-PoIFNα-C2 fusion protein

The pELP-PoIFNα-C2 vector (Fig. 1A) was transformed into *E. coli*, and the expression of fusion protein was induced first with IPTG at 37 °C. SDS-PAGE analysis showed that an expected 75-kDa protein was expressed in the recombinant *E. coli* (Fig. 1B). The fusion protein was expressed mainly as insoluble inclusion bodies since its presence in the insoluble fraction of centrifuged cell lysate. To obtain the soluble fusion

Table 1
Comparison of pharmacokinetics between PoIFNα-C2 protein and PoIFN-α standard in rats.

	Intravenous dosing		Subcutaneous dosing	
	PoIFNα -C2	PoIFN-α	PoIFNα -C2	PoIFN-α
$T_{1/2}$ (h)	56.82 ± 0.70	4.02 ± 0.06	61.58 ± 1.998	6.54 ± 0.16
Cmax (U/ml)	21.98 ± 0.29	9.88 ± 0.04	6.04 ± 0.04	1.84 ± 0.03
$AUC_{0-\infty}$ (h U/ml)	1123.55 ± 4.35	43.82 ± 1.91	743.02 ± 15.63	17.64 ± 4.65
Bioavailability (%)	100.00	100.00	66.13	40.26

protein expression, the protein expression was induced with IPTG at 28, 24 or 20 °C. SDS-PAGE analysis suggested that the solubility of ELP-PoIFNα-C2 fusion protein was temperature-dependent, more than 90% of which was expressed as a soluble protein at 20 °C (Fig. 1C).

3.2. Purification of ELP-PoIFNα-C2 fusion protein

In our pilot experiment, the ELP-PoIFNα-C2 fusion protein was purified with 3 M NaCl at 30 °C as previously described [17]. Although the protein yield was relatively higher, the fusion protein was purified to only 80% purity and difficult to redissolve for further purification by ITC. To reach the compromise between protein yield and purity, we modified the ITC conditions for ELP-PoIFNα-C2 fusion protein purification. First, the ITC was performed with 3 M NaCl at 20, 22, 24, 26, 28 or 30 °C. As expected, the fusion protein precipitation increased gradually with the incubation temperature increase, about 90% of which was precipitated at 28 or 30 °C (Fig. 2A). Then, the ITC was performed with 0.5, 1.0, 2.0 or 3 M NaCl at 28 °C. SDS-PAGE analysis showed that the purity of the fusion protein purified with 2 M NaCl was clearly higher than that with 3 M NaCl (Fig. 2B). However, the purified protein was still difficult to redissolve for next round of ITC. In the light of fact that mild detergents such as Triton X-100 can increase ELP fusion protein solubility without alteration of its phase transition behavior [22], the ITC was performed with 2 M NaCl at 28 °C in the presence of 0.2%, 0.4%, 0.6%, 0.8% or 1.0% Triton X-100. As expected, 0.4% or 0.6% Triton X-100 could increase ELP-PoIFNα-C2 protein solubility without overt alteration of its phase transition behavior (Fig. 2C). Finally, the fusion protein was purified to a single band after two rounds of ITC in the presence of 0.5% Triton X-100. The ITC protocol is summarized as Fig. 3.

3.3. Tag cleavage and target protein recovery

Recently, we produced an active ELK16-TEVp which can be removed simply by centrifugation after tag cleavage [21]. In this study, the TEV protease was used to cleave ELP tag from ELP-PoIFNα-C2 fusion. SDS-PAGE analysis showed that the cleavage efficiency was up to 70% after incubation for 6 h at 30 °C. After removing the protease by quick centrifugation, PoIFNα-C2 protein was recovered by an additional round of ITC with 98% purity (Fig. 4). These data demonstrated the high efficiency of ELP-TEVp system for recombinant protein purification.

3.4. The specific affinity of PoIFNα-C2 protein for pig IgG binding

Recently, we showed that ELP-fused SPG C2 domain can bind to mammalian IgGs and chicken IgY [16]. In this study, the C2 domain was C-terminally fused to PoIFN-α and expressed as a PoIFNα-C2 fusion. Western blotting analysis showed that the fusion protein, but not PoIFN-α standard, could bind to pig IgG (Fig. 5), confirming the specific affinity of recombinant SPG C2 domain for pig IgG binding.

3.5. Antiviral activities of PoIFNα-C2 protein

The antiviral assay showed that both PoIFNα-C2 protein and PoIFN-α standard could inhibit VSV and PRV replication in a dose-dependent manner (Fig. 6). On MDBK cell, PoIFNα-C2 protein showed almost 10-fold lower anti-VSV activity (3.0×10^7 U/μM) than that (2.4×10^8 U/μM) of PoIFN-α standard (Fig. 6A), which may be explained by their difference in molecular weights (25/19 kDa). When tested on PK-15 cells, however, PoIFNα-C2 protein showed higher anti-PRV activity than PoIFN-α standard (Fig. 6B), which may be explained by the relative species specificity of type I IFNs [23] or the increased bioavailability of PoIFNα-C2 protein for PK-15 cells.

3.6. Pharmacokinetics of PoIFNα-C2 protein

The *in vivo* pharmacokinetic analysis showed that the serum half-life of PoIFNα-C2 protein was significantly improved in comparison with that of PoIFN-α standard (Fig. 7). For example, PoIFNα-C2 protein had a $T_{1/2}$ value of 56.82 h (intravenous injection) or 61.58 h (subcutaneous injection), which was 14 or 6 times longer than that (4.02 or 6.54 h) of PoIFN-α standard. The fusion protein had an AUC value of 1124 h U/ml (intravenous injection) or 743 h U/ml (subcutaneous injection), which in each case represented a significant increase over that (44 or 18 h U/ml) of PoIFN-α standard. The PoIFNα-C2 protein had a bioavailability of 66%, which was also significantly improved in comparison with that of (40%) of PoIFN-α standard. The pharmacokinetic comparison between PoIFNα-C2 protein and PoIFN-α standard is summarized in Table 1.

4. Conclusions

In the present study, we demonstrated for the first time that SPG C2 domain can be used to extend half-life extension of PoIFN-α. Since its broad affinity for IgG binding, SPG C2 domain fusion is expected to be a universal strategy for half-life extension of other IFNs.

Acknowledgments

This work was supported by Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pep.2018.08.012.

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