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Short Communication

Soluble Expression, Protein Purification and Quality Control of Recombinant Porcine Interferon-α

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Abstract

Herein, we reported an *Escherichia coli*-based expression and purification method of recombinant porcine interferon alpha (rPoIFN-α). PoIFN-α coding sequence was cloned into pMD18-T vector and then subcloned into pET-32a (+) vector using standard recombinant DNA techniques and the resulting plasmid was transformed into BL21(DE3) competent cells. After induction with isopropyl-β-D-1-thiogalactopyranoside (IPTG), rPoIFN-α was purified from the supernatant of the bacteria lysate using a simple two-step chromatography process consisting of a Ni^{2+} affinity chromatography and a DEAE anion exchange chromatography. rPoIFN-α was purified to >95% homogeneity with a yield of 48 mg/L of culture. It has isoelectic point of 6.09 and bacterial endotoxin was less than 1 EU/mg. N-terminal amino acid sequence and the peptide map digested by trypsin provided additional evidence for the authenticity of rPoIFN-α. The biological activity of rPoIFN-α was 1.1×10^6 IU/mL in HEp-2/ Vesicular Stomatitis Virus (VSV) titration system and its specific activity reached to 1.0×10^6 IU/mg. In conclusion, we obtained high-level expression of a soluble form of bioactive rPoIFN-α by using pET-32a (+) prokaryotic expression system.

Keywords: *Soluble expression, Protein purification, Quality control, Porcine interferon-α, Vesicular Stomatitis Virus (VSV)*

Rekombinant Domuz İnterferon-α'nın Çözünür Ekspresyonu, Protein Saflaştırması ve Kalite Kontrolü

Özet

Bu sunuda rekombinant domuz interferon alfa (rPoIFN-α)'nın *Escherichia coli*-temelli ekspresyonu ve saflaştırma metodu rapor edilmiştir. PoIFN-α kodlayan sekansı pMD18-T vektör içine klonlandı ve sonrasında standart rekombinant DNA teknikleri kullanılarak pET-32a (+) vektör içine subklonlandı ve elde edilen plazmid BL21(DE3) kompetan hücreler içine nakledildi. İzopropil-β-D-1-tiogalaktopiyranosid (IPTG) ile uyarmanın ardından rPoIFN-α, bakteri lizatının süpernatantından basit iki basamaklı kromatografi işlemi (Ni^{2+} affinite kromatografi ve DEAE anyon değişim kromatografi) kullanılarak saflaştırıldı. rPoIFN-α 48 mg/L kültür oluşumu ve >95% homojenite ile saflaştırıldı. Ürün 6.09 izoelektrik puanına sahip olup bakteriyal endotoksin 1 EU/mg'dan daha azdý. N-ucu amino asit sekansý ve tripsin ile oluþturulan peptit haritasý rPoIFN-α'nın özgünlüğü hakkında ilave kanýt saðladý. rPoIFN-α'nın biyolojik aktivitesi HEp-2/ Vesicular Stomatitis Virus (VSV) titrasyon sisteminde 1.1×10^6 IU/mL olarak tespit edilirken spesifik aktivitesi 1.0×10^6 IU/mg'a ulaştı. Sonuç olarak, pET-32a (+) prokaryotik ekspresyon sistemi kullanılarak biyoaktif rPoIFN-α'nın çözünür formunun yüksek derecede ekspresyonu sağlandı.

Anahtar sözcükler: *Çözünür ekspresyon, Protein saflaştırma, Kalite Kontrol, Domuz interferon-α, Vesicular Stomatitis Virus (VSV)*

INTRODUCTION

Among type I Interferons (IFNs), IFN-α plays important roles in inhibition of viral replication [1]. Previously, recombinant IFN-α has been successfully expressed in prokaryotes, eukaryotes and baculovirus [2-4]. However, the function of the *E. coli* expressed products was constrained by protein misfolding [3]. The protein expressed in *Pichia*

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was readily degradable. The baculovirus expression system does not sustain continuous high level expression.

In the present study, we represented the expression, purification, and quality control scheme for producing bioactive rPoIFN-α in large scale. It will facilitate the biological research and clinical application of porcine IFN-α.

MATERIAL and METHODS

Bacterial Strains, Reagents and Cell Lines

Molecular biology reagents were purchased from TaKaRa Biotech (TaKaRa, Dalian, China). The Ni^{2+} His-bind resin and DEAE -Sepharose Cl 6B column were obtained from GE Healthcare (Piscataway, NJ, USA). The mouse anti-PoIFN-α monoclonal antibody was purchased from Abcam (ab11408, Abcam, Cambridge, UK). The pET-32a (+) vector, *E. coli* DH5α, *E. coli* BL21 (DE3), and HEp-2 cell line were preserved in our laboratory.

Porcine IFN-α cDNA Cloning

Total RNA was extracted from peripheral white blood cells of a 6-month-old Bamei pig and was then reverse transcribed to cDNA. The primer sequences for RT-PCR of PoIFN-α (NCBI accessing number AY345969) were 5′-GGAATTCATGTGTGACCTGCCTCAG-3′ (forward) and 5′-CTCGAGTCACTCCTTCTTCCTGAGT-3′ (reverse) which included *EcoR*I and *Xho*I sites (underlined). The amplification length was 501 bp, and It did not include the signal peptide sequence. The RT-PCR product was cloned into pMD-18T vector and the resulted recombinant plasmid was further confirmed by PCR and DNA sequencing, The final product was named as pMD18T-PoIFN-α.

Expression Vector Construction

The inserted PoIFN-α gene in pMD18T-PoIFN-α was digested by *EcoR* I and *Xho* I, and was then ligated into the pET-32a (+) plasmid. The authenticity, orientation and reading frame of the recombinant plasmid pET-32a (+)-PoIFN-α was verified by DNA sequencing.

Expression of PoIFN-α Protein

The plasmid pET-32a (+)-PoIFN-α was transformed into competent *E. coli* BL21 (DE3). The bacteria were cultured in LB medium at 37°C to a density of OD_{600}=0.6. After 4 h induction by IPTG, the bacteria were collected and resuspended in lysis buffer for sonication The lysate was then centrifuged and the supernatant and pellet were collected separately [5].

Purification of rPoIFN-α Protein

The rPoIFN-α protein in the supernatant of cell lysate was purified with *Chelating Sepharose Fast Flow* Ni^{2+} *chromatography* (GE Healthcare, Piscataway, NJ, USA) *following the protocol from the manufacturer.* The chromatogram were shown in *Fig. 2A* and *Fig. 2B*.

Determination of Protein Concentration and Purity

The protein concentration was determined by the Bradford method [6]. Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis was used to determine the purity of the purified rPoIFN-α product. The integrity and specificity of the purified proteins were demonstrated by Western blot assay.

Mass Spectrometry Analysis

The purified protein was further analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS). Mass analysis was performed using a Voyager DE-STR Biospectrometry™ Workstation (Applied Biosystems, Foster City, CA, USA).

Determination of rPoIFN-α Biological Activity

A cytopathic effect inhibition based IFN-α bioassay [7] was used to evaluate the ability of the recombinant protein to protect HEp-2 cells from *VSV* infection. Data were expressed as mean unit (U)/mL, where 1 unit of IFN-α activity was defined as the reciprocal of the dilution producing 50% inhibition of CPE. The titer of sample IFN, was determined by the Reed-Muench method as previously described [8].

Other Quality Control Measurement of rPoIFN-α

The peptide map, isoelectric point, endotoxin, ultraviolet spectroscopy, and N-terminal amino acid sequencing of rPoIFN-α were all determined according to the guidelines in Veterinary Pharmacopoeia of People's Republic of China (2010 edition) [9].

RESULTS

Soluble Expression of Recombinant Protein pET-32a (+)-PoIFN-α

The rPoIFN-α protein was over expressed as shown by a dominant band of 35.0 kDa in Coomassie blue stained PAGE gel *(Fig.1A)*. Besides, the over-expressed protein in the *E. coli* culture was found majorly in the supernatant, not in the pellet *(Fig.1B)*. By SDS-PAGE analysis, the expressed recombinant protein contituted to 32% of the total cellular protein, or 48 mg/L in *E. coli* culture.

Purification of pET-32a (+)-PoIFN-α Protein

In the supernatant of cell lysate, it was shown a single protein peak by Ni^{2+} affinity chromatography *(Fig. 2A)* and by DEAE anion exchange chromatography *(Fig. 2B)*. The result of purification by HPLC showed that there was a dominant protein peak with purity of 95.5% *(Fig.*

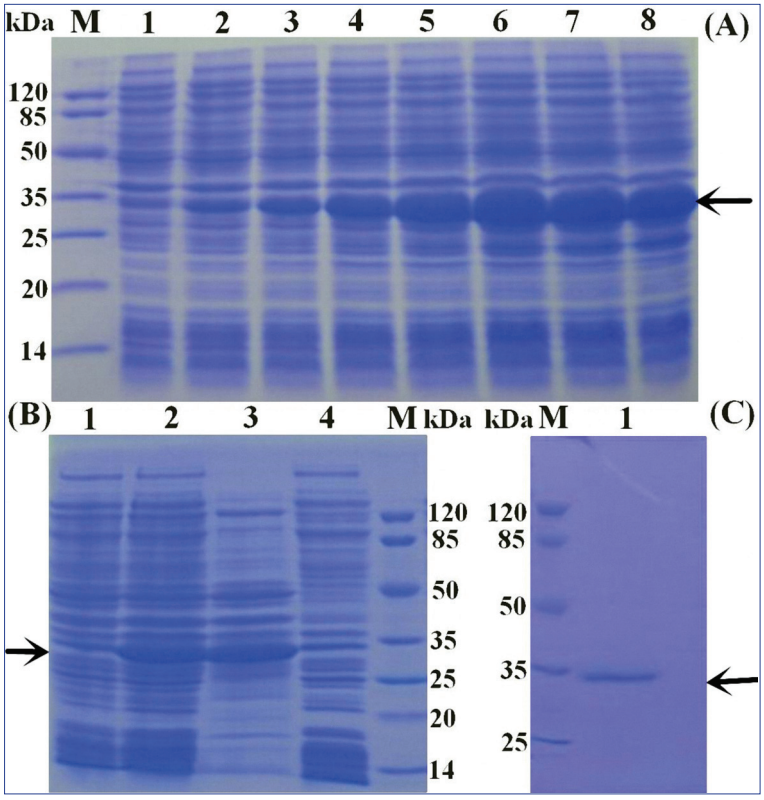

Fig 1. Analysis of rPoIFN-α protein by 12% SDS-PAGE

A: Lane M: molecular weight marker. Lane 1-8. Total cellular proteins from *E. coli* BL21(DE3) at 0h, 1h, 2h, 3h, 4h, 6h, 8h, 10h after IPTG induction, **B:** Lane 1. Total proteins before induction. Lane 2. Total proteins after induction. Lane 3 Supernatant of bacterial lysate after ultrasonication. Lane 4. Pellet of bacterial lysate after ultrasonication**.** Lane M: molecular weight marker, **C:** Lane M. Molecular weight marker. Lane 1. Purified rPoIFN-α after Ni^{2+} affinity chromatography and DEAE anion exchange chromatography

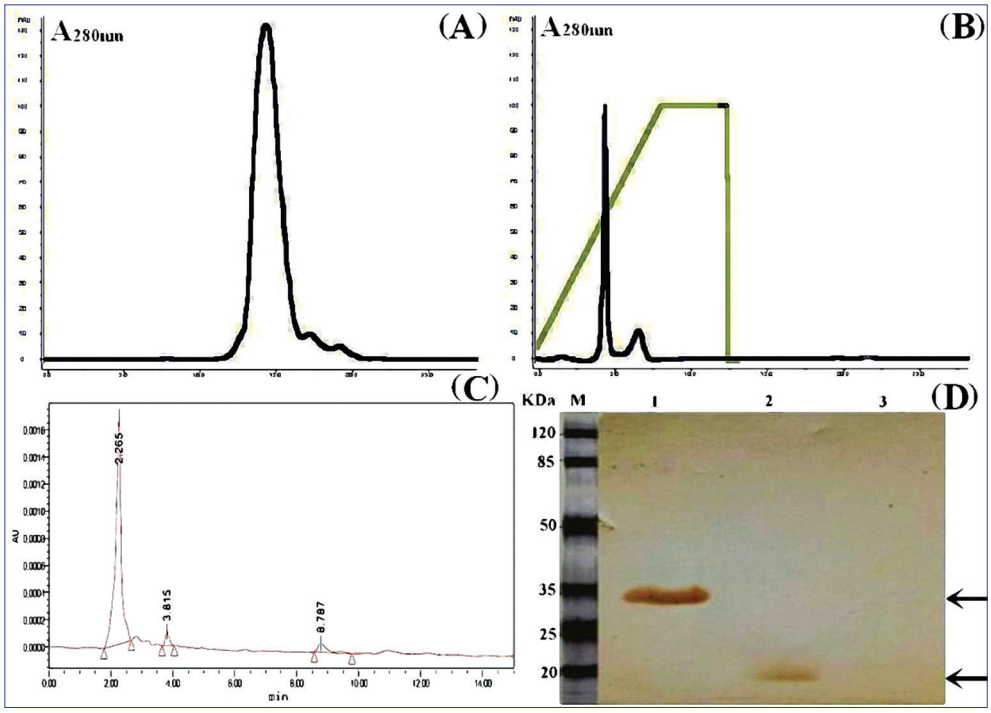

Fig 2. Protein characterization by affinity chromatography, anion exchange chromatography, HPLC and Western blot

A: Ni^{2+} affinity chromatography with absorbance at 280nm, **B:** DEAE anion exchange chromatography with absorbance at 280 nm, **C:** HPLC examination of purified proteins. The peak is the target protein which was detected at a wavelength of 280 nm, **D:** Western Blot analysis of purified proteins. Lane M. Molecular weight marker. Lane 1. Purified rPoIFN-α protein without enterokinase digestion. Lane 2. Purified rPoIFN-α with enterokinase digestion. Lane 3. Total cellular proteins before induction

2C). Western blot analysis with anti-PoIFN-α monoclonal antibody showed a non-enterokinase digested protein product at 35 kDa *(Fig. 2D)* and a enterokinase digested PoIFN-α protein at 19.3 kDa *(Fig. 2D)*, consistent with that in SDS-PAGE gel *(Fig. 1C)*.

The purification chart of rPoIFN-α from 300 mL of bacterial culture showed that the recombinant rPoIFN-α was purified to 4.9 fold by the two-step purification procedure and its specific activity reached to 1.0×10^6 IU/mg *(Table 1-A)*.

Bioactivity of Purified rPoIFN-α

The results showed that HEp-2 cells pretreated with 1 U of purified rPoIFN inhibited 50% of *VSV* infection

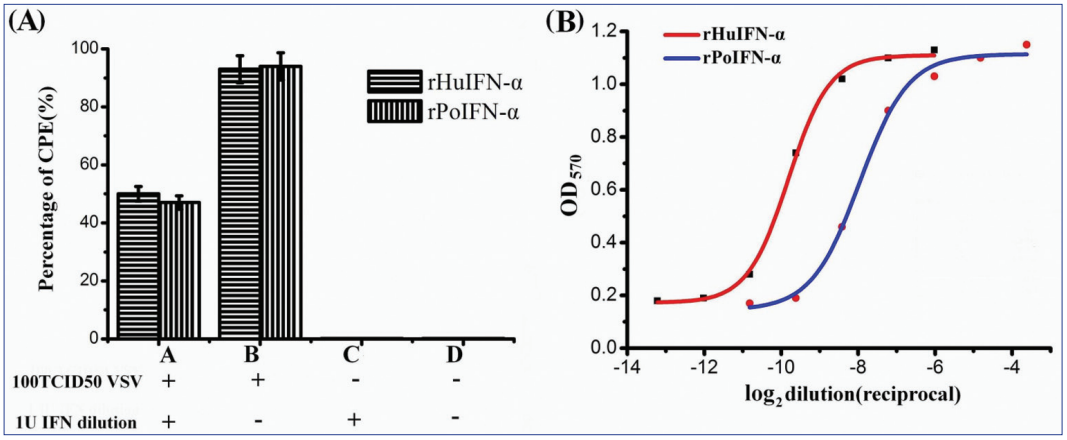

Fig 3. Antiviral activity of the rPoIFN-α in HEp-2/*VSV* titration system

A: 50% of CPE inhibition by 1 unit of rPoIFN-α and 1 unit of rHuIFN-α and the control groups for the titration of biological activity of IFN, **A:** about 50% CPE was observed in *VSV* infected cells pre-incubated with 1 unit of IFN-α, **B:** about 90% CPE was observed in *VSV* infected cells without IFN-α treatment, **C:** No CPE was observed in the cells pre-incubated with 1 unit of IFN-α without *VSV* infection, **D:** No CPE was observed in the cells which was treated with neither *VSV* infection nor IFN-α addition. **B:** The dose-response curve of interferon in HEp-2/*VSV* system. The figure shows that the titre of rHuIFN-α is slightly higher than that of rPoIFN-α in human cells

Table 1-A. *Purification chart of rPoIFN-α from 300 mL of bacterial culture**

Purification Step	Total Protein (mg)	Total Activity (IU)	Specific Activity (IU/mg)	Fold of Purification
Before purification (Cell Lysate)	77.9	1.6×10^7	2.0×10^5	1.0
After two-step purification	14.5	1.4×10^7	1.0×10^6	4.9

** Results were representative of three independent experiments*

Table 1-B. *Quality control of the bulk of rPoIFN-α*

Category	Method	Specification	Reference
Specific activity	HEp-2/*VSV*	≥1.0×10^6 IU/mg	
Purity	SDS-PAGE and HPLC	≥95.0%	Ref. [9], Appendix 36,41
Bacterial endotoxin	LAL(Limulus Amebocyte Lysate)	<1 EU/mg	Ref. [9], Appendix 130
Isoelectic point	Isoelectrofocusing	6.09(within 4.5~6.5)	Ref. [9], Appendix 41
UV maximum	UV scan	(278±3) nm	Ref. [9], Appendix 26
Peptide map	Tryptic digestion	Conformed to reference	Ref. [9], Appendix 107
N-terminal amino acid sequence	Edman degradation	CDLPQTHSLAHTRAL	Ref. [9], Appendix 32

(Fig. 3A). The antiviral activity of the final rPoIFN-α protein was determined as 1.1×10^6 IU/ml by the bio-activity assay. The inhibitory activity of rPoIFN-α on *VSV* replication in culture was dose dependent. The dose-response curve of interferon in HEp-2/*VSV* system was shown in *Fig. 3B*.

Study on Quality Control of rPoIFN-α

The primary structure of purified rPoIFN-α was confirmed by N-terminal sequencing and Mass Spectrometry analysis *(Table 1-B)*. Also, the recombinant molecules appeared to be homogenous by reversed-phase HPLC analysis and gel filtration *(Fig. 2C)* with no signs of aggregation (data not shown). The results of rPoIFN-α analysis of quality control are summarized in *Table 1-B*.

DISCUSSION

In the production of recombinant protein in heterologous expression systems, solubility is a key issue. Soluble recombinant proteins are usually properly folded, functional and they are much easier to be purified than aggregated proteins obtained from inclusion bodies.

The pET is one of the most powerful systems yet developed for the expression of the recombinant proteins in *E. coli*. The pET32 series were fused with the 109 amino acid Trx•Tag™ thioredoxin protein which is a solubilization tag that assists in the proper folding of the expressed peptides and keeps them from precipitating. This vector also contains cleavable His•Tag® and S•Tag™ sequences for detection and purification. Through the use of combination

of pET-32a (+) vector and BL21(DE3) host cell, the desired expression product can comprise more than 30% of the total cell proteins in a few hours after induction [5].

In summary, the present study demonstrated that a functional porcine IFN-α protein was expressed in *E. coli* in a soluble form. The recombinant protein was readily purified by a two-step chromatographic procedure. Its authenticity and bioactivity were verified by multiple tests of quality control. This protein could be further expected for mass production and clinical applications of rPoIFN-α.

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