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Full Length Research Paper

Preparation and PEGylation of recombinant human interferon lambda3

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The purpose of this study was to express recombinant human interferon lambda3 (rhIFN-λ3) in *Escherichia coli*, and prepare PEGylated recombinant human interferon lambda3 (PEG-rhIFN-λ3). The *rhIFN-λ3* gene was inserted into pThioHisA vector after codon optimization and transformed into *E. coli* top10 strain, and then it was induced with isopropyl-β-D-thio-galactoside (IPTG). The recombinant protein was subjected to mPEG-ButyrALD modification after dialysis, renaturation and chromatographic purification. Subsequently, the modified product was preliminary isolated and purified for determining its activity. Results show that the recombinant protein was expressed in the form of inclusion bodies. After ion exchange, molecular sieve and other column chromatography purification, the purity of the purified rhIFN-λ3 was as high as 90% and the purity of the mono-PEGylated rhIFN-λ3 after cation-exchange chromatography was as high as 86%. The 50% effective concentration (EC_{50}) of rhIFN-λ3 in WISH cells against vesicular stomatitis virus (VSV) was 8.43 ng/mL, while the EC_{50} of mono-PEGylated rhIFN-λ3 was 49.19 ng/mL, which reserved 17.14% of the *in vitro* activity and supported further studies of this new type of investigational interferon. Further study is needed to better understand the *in vivo* immunogenicity, antigenicity, stability and antiviral activity of PEG-rhIFN-λ3.

Key words: Recombinant human interferon lambda3, prokaryotic expression, purification, mPEG-ButyrALD, antiviral activity.

INTRODUCTION

Interferons (IFNs) are a group of cytokines showing antiviral, anti-tumor, antiproliferative, immunomodulatory and other biological activities which play very important functions in inherent immunological and adaptive immunological system. According to the differences in amino acid sequences and receptors, IFNs are classified into type I, type II and type III interferons (Pestka et al., 2004). Type I IFNs contain IFN-α, IFN-β and several other subtypes, and all of the signaling pathways are transduced by IFN-α/β receptor, while the signaling pathway of type II IFN includes only IFN-γ and its receptor. Type III IFNs are a novel group of IFNs that have recently been discovered independently by two research teams (Sheppard et al., 2003; Kotenko et al., 2003). Initially, these cytokines were named as IFN-λ (IFN-λ1, -λ2 and -λ3) or interleukin (IL)-29, -28A and -28B.

So far, both type I and II IFNs have been developed as pharmaceuticals and clinical indications, including interferon alfa-2a (roferon a®), alfa-2b (intron a®, viraferon®, viraferonpeg®), alfacon-1 (infergen®), beta 1a (avonex®, rebif®), beta-1b (betaferon®, extavia®, betaseron®, ziferon®), alfa-n3 (alferon n injection®), gamma-1b (actimmune®) (Peter et al., 2012). These drugs have been approved by the Food and Drug Administration (FDA) or European Union and successfully applied to the treatments on some tumors and viral

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Abbreviations: rhIFN-λ3, Recombinant human interferon lambda3; **PEG-rhIFN-λ3,** PEGylated recombinant human interferon lambda3; **IPTG,** isopropyl-β-D-thio-galactoside; **VSV,** vesicular stomatitis virus; **EC_{50},** 50% effective concentration.

diseases in clinic, such as chronic viral hepatitis, multiple sclerosis, chronic myeloid leukemia, myeloma, asthma exacerbations, chronic granulomatous disease and osteopetrosism (Peter et al., 2012). However, there is a significant side effects profile associated with IFNs. Main adverse effects are influenza-like (fatigue, headache, fever and rigor), psychiatric (depression, irritability and insomnia), hematologic (anemia, neutropenia and trombopenia) and gastrointestinal (nausea, diarrhea) (Ferenci, 2011; Simona and Costin, 2011). Additionally, these drugs require dosing either once daily or three times weekly, for the Tmax of IFNs are approximately 7 to 10 h with drug levels detectable for approximately 30 h *in vivo* (Wills, 1990; Peter et al., 2012). Conjugation of IFNs with polyethylene glycol (PEG) increases efficacy through increasing solubility and decreasing antigenicity, proteolysis and renal clearance (Pasut and Veronese, 2007). Therefore, two PEGylated formulations of IFN alfa (Zeuzem et al., 2003): PEGylated IFN alfa-2a (pegasys®) and PEGylated IFN alfa-2b (pegintron®) have been approved for use in the United States and the European Union. Nevertheless, the two products seem to be comparable in terms of adverse effects that are similar to usual IFNs, leading to treatment discontinuation.

It has been confirmed that type III IFNs display similar properties to type I IFNs, signaling through the JAK-STAT pathway and up-regulating the expression of genes involved in controlling viral replication and cellular proliferation (Sheppard et al., 2003; Witte et al., 2010). However, type III IFNs has a complex binding, the IL28 receptor, which is distinct from that used by type I IFNs, and is present only on plasmacytoid dendritic cells, peripheral B cell epithelial cells and hepatocytes (Pestka et al., 2004; Kotenko, 2011). This restricted receptor distribution offers type III IFNs a better tolerability and safety profile, especially in terms of bone marrow suppression (Witte et al., 2009), indicating that type III IFN can be developed as an alternative to type I IFNs or used in combination (Miller et al., 2009), thus enhancing the sub-saturating levels of type I IFNs and providing additive therapeutical effects (Pagliaccetti et al., 2008) and improving side-effect profiles. IFN-λ1 has been PEGylated and tested in Phase IIb (ZymoGenetics/ Bristol-Myers squibb). Preclinical and clinical data showed that PEGylated IFN-λ1 has antiviral activity against hepatitis C virus (HCV) but have fewer side effects (Muir et al., 2010; Ramos, 2010). Recently, a single nucleotide polymorphism (SNP) upstream of the *IL-28* gene (encoding for IFN-λ3) has been correlated with spontaneous clearance of the infection and success of therapy (Ge et al., 2009; Tanaka et al., 2009). Although these results argue that IFN-λ3 might play a major role in controlling HCV, the molecular mechanism remains to be determined. So we are interested in IFN-λ3 and want to investigate this new type of IFN.

In the present study, human *IFN-λ3* gene was optimized based on the codon bias of *Escherichia coli* and expressed in the *E. coli* top 10 strain. The recombinant protein was refolded by dialysis, purified with chro-matographic method and modified by methoxy-polyethylene glycol-butyraldehyde (mPEG-ButyrALD). Furthermore, the antiviral activities of rhIFN-λ3 and PEGylated rhIFN-λ3 were compared with commercially available rhIFN-α2a with cytopathic inhibition assay. The present study established the basis for further studies on IFN-λ modification with PEG.

MATERIALS AND METHODS

Plasmids, strains and cell lines

The plasmids pEASY-Blunt Smiple and PThioHisA were used as cloning and expression vector, respectively. *E. coli* DH5α and Top10 strains were used for hosts in this study. Indiana strain of VSV virus was previously stored in our lab; the WISH cell lines were purchased from Cell Resource Center, IBMS, CAMS/PUMC.

Reagents and media

The DNA marker, LMW protein marker and restriction endo-nucleases were purchased from Takara Corporation. Q Sepharose HP, CM Sepharose FF and Superdex 75 column were purchased from Amersham Pharmacia Biotech Corporation. mPEG-ButyrALD (Mr: 10 kDa) was purchased from Beijing Kaizheng Biotech Development Company Limited, IL-28B/IFN-λ3 monoclonal antibody was purchased from R and D Systems, hIFN-α2a was purchased from PeproTech Corporation. *E. coli* strains were cultured in Luria-Bertani (LB) medium which was purchased from Sigma Corporation. WISH cells were cultured in Dulbecco's modified eagle's medium (high glucose) purchased from Gibco Corporation. Other chemicals were of analytical grade and commercially available.

rhIFN-λ3 gene optimization and synthesis

According to the codon bias of *E. coli*, human *IFN-λ3* gene was optimized with reference to the mature human IFN-λ3 cDNA (accession number NM_172139). Additional EcoRI and SalI sites were added to the 5' and 3' ends of the gene, respectively. Optimized *IFN-λ3* gene was synthesized by Beijing Sunbiotech Corporation and inserted into a cloning vector pEASY-Blunt Smiple.

Construction of the rhIFN-λ3 expression vector

The recombinant vector pEASY-Blunt-rhIFN-λ3 and pThioHisA vector were digested with EcoRI and SalI, recovered and then ligated with T4 DNA ligase. The ligation mixture was transformed into *E. coli* DH5a. Subsequently, a colony was cultured and the recombinant plasmid was extracted and digested with EcoRI and SalI for identification. Finally, it was confirmed by polymerase chain reaction (PCR) and sequencing.

Protein expression and optimization

The confirmed pThioHisA-rhIFN-λ3 was transformed into the competent *E. coli* top10 cells to generate the stable expression line. A single colony of the transformant was inoculated into 5 mL LB medium containing 100 μg/mL ampicillin and incubated overnight

with vigorous shaking (200 rpm) at 37°C. 100 microliters of culture was transferred to 5 ml fresh LB medium in a 10 mL tube. The culture was incubated in 200 rpm at 37°C until the OD_{600} reached 0.4 to 0.6 and then 5 μL 100 mM isopropyl-β-D-thio-galactoside (IPTG) was added for induction. One milliliter of the sample was collected at 4 h after induction and analyzed by 14% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, induction time, temperature and IPTG concentration, and other factors that may influence the final protein expression were optimized.

Protein denaturation, refolding and purification

After incubation, the cells were harvested by centrifugation and resuspended in lysis buffer A (50 mmol/L Tris-HCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1.5% sucrose, 100 mmol/L NaCl, 0.5% Trition X-100 (V/V), pH 8.0) and lyzed by sonication. The cell debris was collected by centrifugation (12000 *g* for 30 min) and rinsed with buffer B (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 2 mol/L urea, pH 8.0). Then, the inclusion bodies were collected by centrifugation and solubilized in buffer C (8 mol/L urea, 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 10 mmol/L dithiothreitol, pH 8.5). Following centrifugation at 12000 *g* for 20 min at 4°C, the supernatant containing the proteins was collected and loaded onto an ion-exchange Q-Sepharose HP packed column which was pre-equilibrated with buffer C. Subsequently, the column bound proteins were eluted using a programmed gradient of NaCl in buffer C. Finally, SDS-PAGE eletrophoresis was carried out for the purified proteins.

For denaturation, the purified protein was diluted by two-fold with the refolding buffer D (20 mmol/L Tris-HCl, 200 mmol/L NaCl, 2 mmol/L reduced glutathione, 0.5 mmol/L oxidized glutathione, 5% glycerol, pH 8.2) supplemented with 6 mol/L urea and dialyzed against buffer D supplemented with 2 mol/L urea for 5 h. Next, they were dialyzed against buffer D overnight, and finally the precipitation was removed by centrifugation. Following refolding, the supernatant containing the proteins were dialyzed against buffer E (20 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$, 5% glycerol, pH 7.4) for 6 h. Then, enterokinase (EK) was added in 1:150 (V/V) for another 12 h to cleave off the thioredoxin A tag (HP). The mixture was loaded onto an ion-exchange CM-Sepharose fast flow (FF) packed column which was pre-equilibrated with buffer E and then eluted using the programmed gradient of NaCl in buffer E. Fractions containing the protein were pooled and then loaded onto Superdex 75 column. The protein was eluted with size exclusion buffer F (20 mmol/L Na_2HPO_4-KH_2PO_4, pH 6.5). Finally, the purity was determined by 14% SDS-PAGE electrophoresis.

Western blot analysis

For western blotting, the proteins were transferred to polyvinylidene fluoride (PVDF) membrane on a tank-blotting device at 9 V for 60 min. Then, the membrane was blocked at room temperature for 2 h with 5% (m/v) milk powder diluted in phosphate buffered saline (PBS), and the membrane was incubated at 37°C for 1 h in the presence of the rhIFN-λ3 monoclonal antibody (2 ug/mL). After washing the membrane, peroxidase-conjugated affinipure goat anti-mouse IgG (H + L) antibody (diluted at 1:5000) was added and incubated at 37°C for 1 h. Finally, the membrane was washed and the membrane was stained using the peroxidase substrate 3, 3'-diaminobenzidine (DAB).

Protein PEGylation and purification

The purified rhIFN-λ3 protein was dialyzed against buffer G (50 mmol/L Na_2HPO_4-KH_2PO_4, 5 mmol/L sodium cyanoborohydride, pH 6.5) overnight and then PEGylated with mPEG-ButyrALD. To optimize the reaction conditions, the rhIFN-λ3 was incubated with varying amounts of 10 kDa mPEG-ButyrALD in buffer G over a pH range from 4.0 to 9.0 and at an ambient temperature from 4 to 37°C for 0 to 24 h. Next, PEGylated rhIFN-λ3 was generated according to the optimized PEGylation procedure and used as the initial material for purification. The reaction mixture was diluted with buffer H (20 mmol/L PB, pH 6.5) and loaded onto an ion-exchange CM-Sepharose FF packed column which was pre-equilibrated with buffer H, and then eluted using a programmed gradient of NaCl in Buffer H. Finally, Lowry method was used to determine the total protein quantity.

Antiviral assays

According to the cytopathogenic effect (CPE) inhibition assay in Chinese Pharmacopoeia (2010 Edition), the capability of rhIFN-λ3, PEG-rhIFN-λ3 and commercially available rhIFN-α2a in protecting WISH cells from lysis by VSV was measured. Briefly, WISH cells were inoculated in a 96-well microtiter plate and treated with indicated amounts of IFN for 24 h and then challenged with VSV for 20 h. Each concentration of IFN was repeated four times in one plate and the status of cell survival was measured by a crystal violet coloration assay. One control group was left untreated (no IFN and no virus) and another control group was infected with the virus without treating with IFN. The measured absorbance in the cytokine wells (Abs) were normalized as a percentage of the cell control (CC) with the virus control (VC) subtracted, using the following equation: normalized cell survival (NCS) = (Abs - VC) / (CC - VC) × 100 to allow comparisons in the activities among plates. EC_{50} and exponential slop values were calculated by using SPSS 12.0 software.

RESULTS

Identification of recombinant plasmid pThioHisA-rhIFN-λ3

For expressing *IFN-λ3* gene, the recombinant plasmid pThioHisA-rhIFN-λ3 was constructed. The recombinant plasmid map is shown in Figure 1. The results from agarose gel electrophoresis showed that the recombinant plasmid pThioHisA-rhIFN-λ3 was successfully constructed and was further confirmed by using two restriction endonucleases (EcoRI/SalI) and PCR methods (Figure 2). The result of sequencing for the DNA fragment was in accordance with the designed *rhIFN-λ3* gene.

Optimization of expression conditions

The *E. coli* top10 cells transformed with pThioHisA-rhIFN-λ3 plasmid were cultured and induced with IPTG to express the fusion protein HP-rhIFN-λ3. The bacteria were collected before and after IPTG induction for SDS-PAGE analysis. A band of approximately 33 kDa was observed in the IPTG-induced cells, which were close to the calculated molecular weight of HP-rhIFN-λ3. By optimizing the expression conditions, high level of HP-rhIFN-λ3 was found after induction with 1 mM IPTG for 6

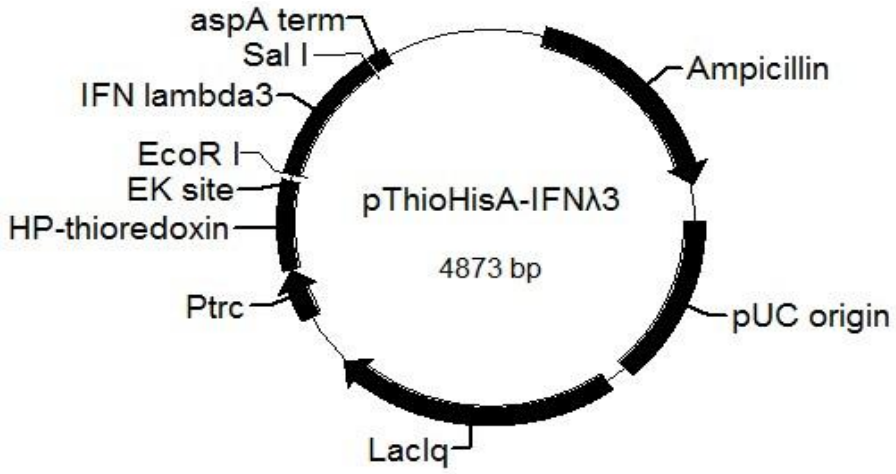

Figure 1. Schematic diagram of expression construct.

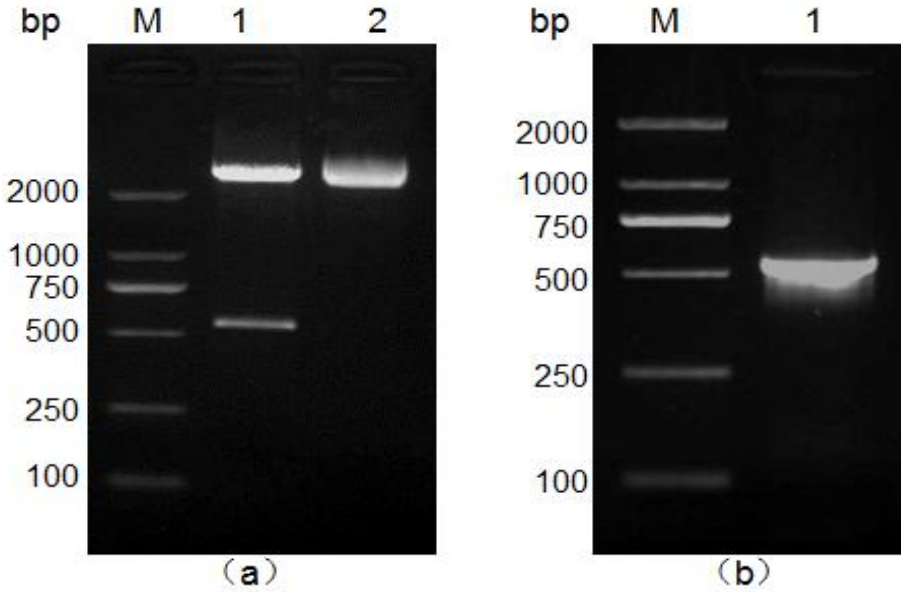

Figure 2. Identification of pThioHisA-rhIFN-λ3 by enzymatic digestion and PCR method. (a) Identification of the recombinant plasmid pThioHisA-rhIFN-λ3 by enzymatic digestion. M, DNA molecular standard DL2000; 1, recombinant plasmid pThioHisA-rhIFN-λ3 digested with EcoRI/SalI; 2, recombinant plasmid pThioHisA-rhIFN-λ3. (b) PCR analysis for *rhIFN-λ3* gene. M, DNA molecular standard DL2000; 1, PCR product of rhIFN-λ3.

h (Figure 3), and the fusion protein HP-rhIFN-λ3 accounted for more 31% of the total proteins.

Protein purification

After IPTG induction, the bacteria harboring HP-rhIFN-λ3 was sonicated, and most of the HP-rhIFN-λ3 was detected in the inclusion bodies as measured by the densitometry scanning for the stained gel (Figure 4a). As shown in Figure 4b, the purified HP-rhIFN-λ3 and hIFN-λ3 were successfully obtained by chromatography.

Western blot analysis

Western blot was carried out with rhIFN-λ3 antibody. Two bands at about 33 and 19 kDa corresponding to HP-

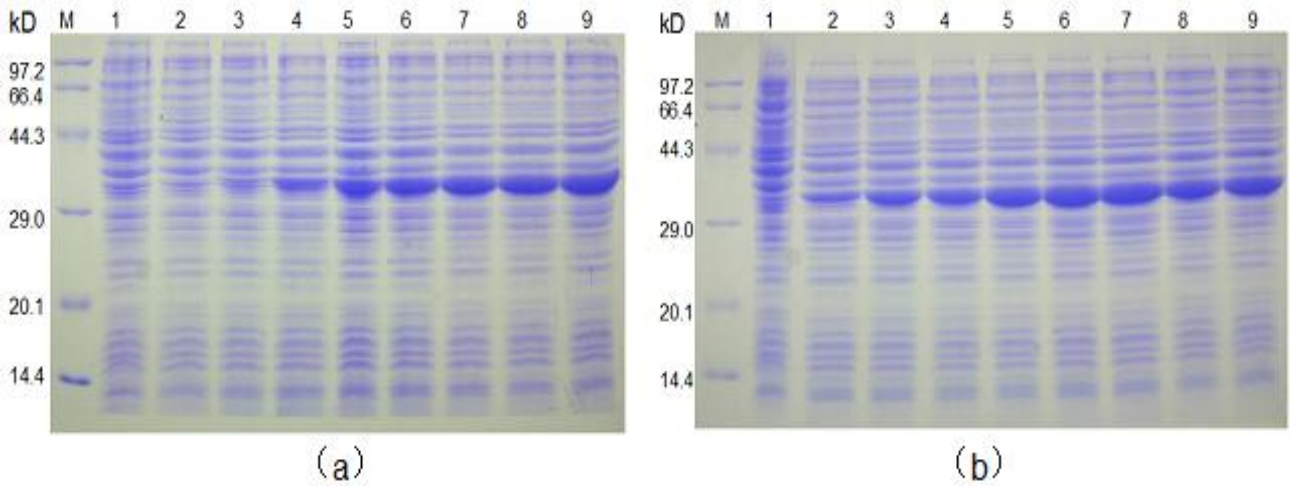

Figure 3. SDS-PAGE analysis for HP-rhIFN-λ3 expression. (a) Induced with different concentrations of IPTG. M, molecular weight marker; 1, non-induced bacteria; 2 to 9, bacteria induced with 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 mmol/L IPTG, respectively. (b) Expression at different time points. M, molecular weight marker; 1, total cell lysate of top10 control; 2 to 9, total cell lysate of top10 cells expressing fusion protein at 1, 2, 3, 4, 5, 6, 7 and 8 h, respectively.

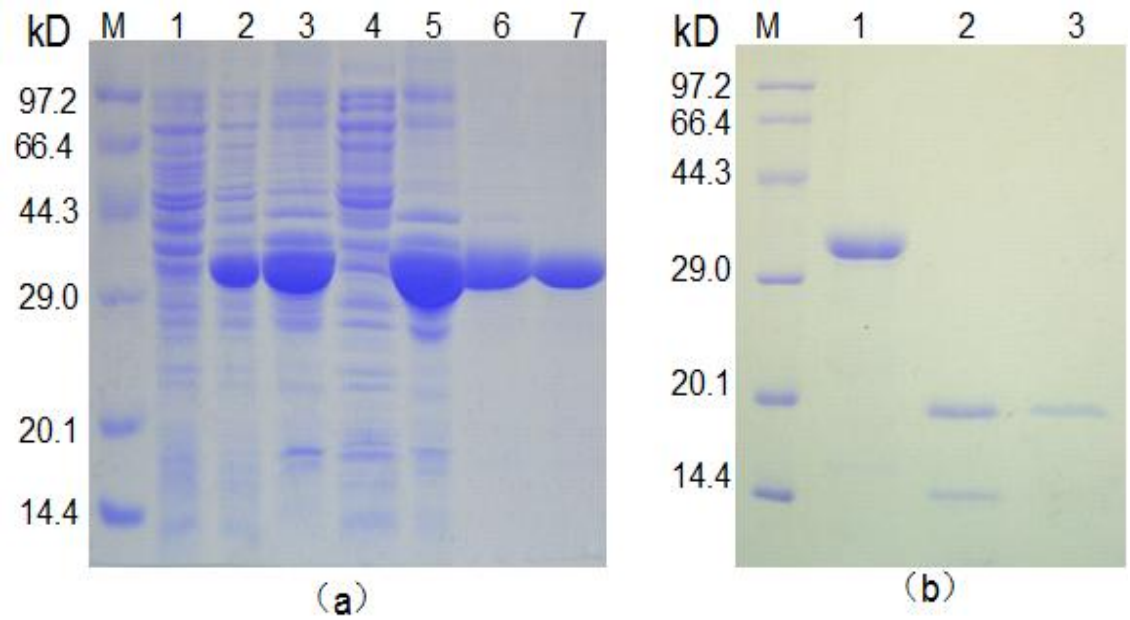

Figure 4. Purified HP-rhIFN-λ3 and hIFN-λ3. (a) Purified HP-rhIFN-λ3 inclusion bodies. M, Molecular weight marker; 1, non-induced bacteria; 2, induced bacteria; 3, precipitation after sonication; 4, supernatant after sonication; 5, sample from inclusion bodies; 6, target product purified by Q Sepharose HP; 7, HP-rhIFN-λ3 protein from renaturation. (b) Purified HP-rhIFN-λ3 and hIFN-λ3. M, Molecular weight marker; 1, target product purified by Q Sepharose HP; 2, EK cleavage of HP-rhIFN-λ3 protein; 3, rhIFN-λ3 from Superdex 75.

rhIFN-λ3 and rhIFN-λ3 were detected in the membrane (Figure 5). The western blot result supported that the observed induced fusion protein was HP-rhIFN-λ3 and the obtained rhIFN-λ3 with a molecular weight of 19 kDa was a product by enterokinase (EK) cleavage.

PEGylation and purification

The final conditions for PEGylation were as follows: rhIFN-λ3 and 10 kDa mPEG2-butyrALD in molar ratio of 1:10 at pH 6.5 and 37°C for 8 h in the phosphate buffer.

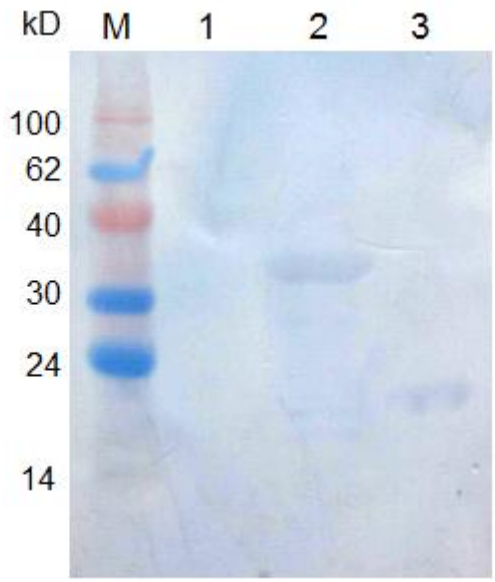

Figure 5. Western blot for purified HP-rhIFN-λ3 and rhIFN-λ3. M, Prestained molecular weight marker; 1, non-induced bacteria; 2, purified HP-rhIFN-λ3; 3, purified rhIFN-λ3.

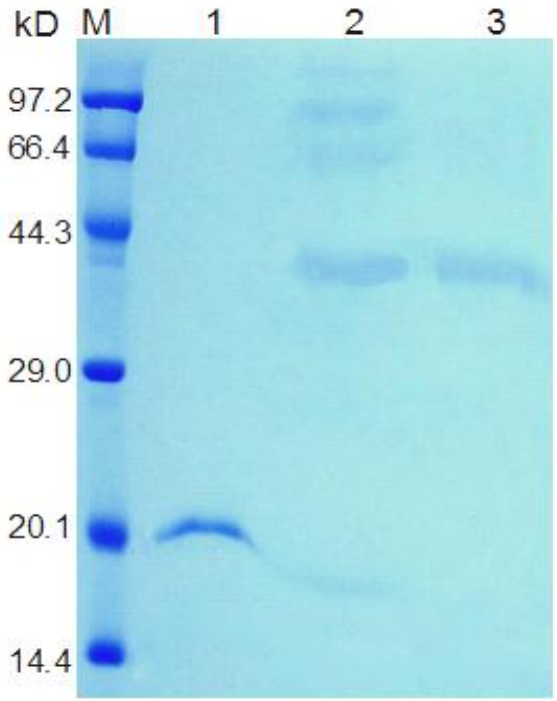

Figure 6. Purified mPEG-rhIFN-λ3. M, Molecular weight marker; 1, purified rhIFN-λ3; 2, mixture of mPEG-rhIFN-λ3; 3, purified mPEG-rhIFN-λ3.

PEGylated rhIFN-λ3 generated according to the optimized PEGylation procedure was used as the initial material for purification. After the CM Sepharose FF chromatography, a band of mono-PEGylated rhIFN-λ3 for about 40 kDa was obtained while the uncoupled PEG, free rhIFN-λ3 and multiple-modified rhIFN-λ3 were removed (Figure 6).

Determination of antiviral activity

To measure the activity of the purified protein, an assay was established in which the ability of rhIFN-λ3 to protect WISH cells from lysis by VSV was measured. The rhIFN-λ3 showed potency in WISH cells against VSV with half-maximal effective concentration (EC_{50}) after codon optimization was higher than that for the positive control rhIFN-α2a, indicating that the antiviral activity of rhIFN-λ3 was lower than that of rhIFN-α2a (Figure 7). The anti-viral activity of PEGylated rhIFN-λ3 reserved 17.14% of the activity of IFN-λ3 before modification (Table 1).

DISCUSSION

IFN-λ has both *in vitro* and *in vivo* antiviral activity as type I IFN (Witte et al., 2010). The similar biological effects caused by type I and III IFNs can be explained by the activation of a highly overlapped set of transcription factors. Type I and III IFNs all take effects through the receptor-associated tyrosine kinases JAK1/TYK2 which mediate the phosphorylation of transducers and activators for transcription (Gad et al., 2009; Dellgren et al., 2009). However, unlike the widely distributed IFN-a receptor, IFN-λ receptor expression is more restricted. The limited distribution of the IFN-λ receptor suggests that the potential for reducing adverse events in IFN-λ–based therapy in comparison to IFN-a-based therapy is concurrent with the preservation of the antiviral effect in hepatocytes diseases (Muir et al., 2010).

E. coli is the dominant host for producing protein pharmaceuticals, which offers a means for rapid and economical production of recombinant proteins (Swartz et al., 2001). When comparing the different codons of hIFN-λ3 from GenBank with those of *E. coli*, we found that the hIFN-λ3 used several rare codons of *E. coli,* including eight arginine codons, five proline codons, and one isoleucine codon. To improve the expression efficiency of hIFN-λ3, the 14 codons were optimized according to the codon bias of *E. coli*. Our data shows that hIFN-λ3 was expressed in high level, in the form of inclusion bodies in *E. coli*. To evaluate the conditions affecting solubility, disulfide-bond formation and isomerization, several refolding strategies were tried, and it was found that dialysis was the optimal refolding method for hIFN-λ3. Out of seven cysteines present in hIFN-λ3, six cysteines were engaged in intramolecular disulfide-bonds (Gad et al., 2009). The utilization of glutathione can improve the formation of disulfide-bonds. We purified the protein by ion-exchange and size-exclusion chromatography, and a

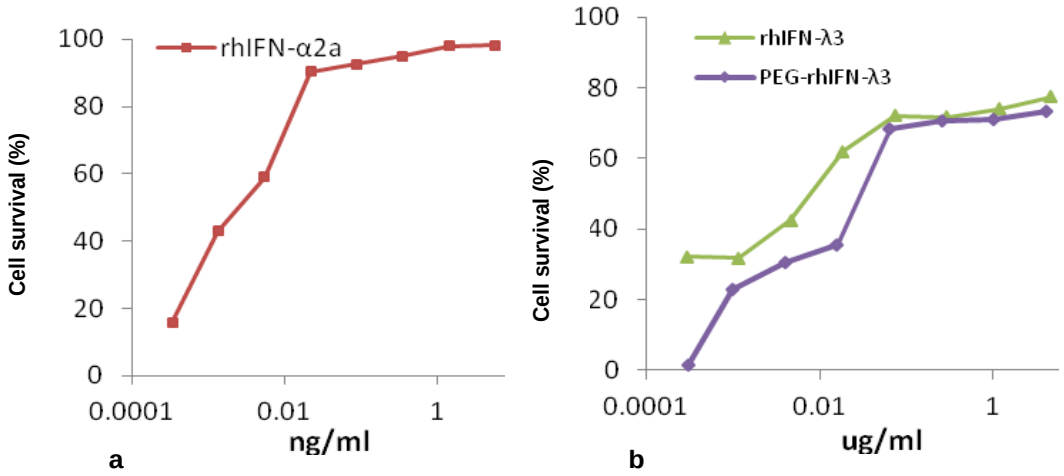

Figure 7. Antiviral effect of IFN concentrations in WISH cells challenged with VSV. WISH Cells were incubated with serial dilutions of IFN before being challenged with VSV. (a) rhIFN-α2a purchased from PeproTech. (b) Refolded rhIFN-λ3, purified PEG-rhIFN-λ3.

Table 1. Antiviral activity on WISH cells *in vitro.*

Protein	EC_{50}
rhIFN-α2a	2.38 pg/mL
rhIFN-λ3	8.43 ng/mL
PEG-rhIFN-λ3	49.19 ng/mL

highly pure product was obtained with a purity higher than 90%. The purified rhIFN-λ3 showed a weaker anti-VSV activity than PeproTech rhIFN-α2a.

To prolong the duration of action, IFN was conjugated with PEG. PEGylated type I IFNs have been well established and widely used in clinical applications. PEGylated IFNs also increase the apparent molecular weight of the polypeptide, thus reduce renal clearance rate and alter biodistribution. Here, rhIFN-λ3 was PEGylated with mPEG-ButyrALD, which is mainly used to achieve PEGylation at the N-terminus. SDS-PAGE analysis of the reaction mixture showed that there were significant amount of monoPEGylated species in the reaction mixture, while other three bands present above the band of interest were observed. This implies conjugation of sites other than the N-terminus. In theory, the preferential site will be occupied first, but as high PEG equivalent was used to drive the reaction, other amine sites will be occupied after N-terminus has been occupied, thus multiPEGylated rhIFN-λ3 was gained. Therefore, we have optimized the reaction conditions and developed an efficient purification procedure for site-specific PEGylation of rhIFN-λ3. A cation exchange chromatography was developed to remove the unreacted PEG, the uncoupled rhIFN-λ3 and the multiple modified rhIFN-λ3. The purity of single PEGylated rhIFN-λ3 was as high as 86%. Additionally, the apparent molecular weight (40 kDa) of PEG-rhIFN-λ3 on the gel was much greater than the theoretical molecular weight (29 kDa), which can be explained by the increased hydrodynamic volume of the PEGlated protein. It resulted from the ability of PEG in coordinating water molecules and the high flexibility of PEG chain (Basu et al., 2006). Our data shows the mono-PEGylated rhIFN-λ3 reserved 17.14% of the activity in comparison to the *in vitro* activity of rhIFN-λ3. This might be explained by the fact that PEG chains can sweep around the protein to shield and protect it from the environment, but they also influence the interactions of the protein that were responsible for its biological functions (Simona et al., 2010). Therefore, the preserved *in vitro* biological activity after PEGylation was reduced, sometimes very significantly; nevertheless, the *in vivo* pharmacological effects were usually enhanced.

In summary, the expression plasmid pThioHisA-rhIFN-λ3 was successfully constructed and expressed at a relatively high level in *E. coli*. PEGylation and purification process were carried out to obtain the mono-PEGylated rhIFN-λ3. Moreover, the *in vivo* immunogenicity, antigenicity, stability and antiviral activity of PEG-rhIFN-λ3 still need further studies.

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