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Production of Recombinant Porcine Interferon alpha Using PHB–Intein-Mediated Protein Purification Strategy

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Abstract Interferons (IFNs) are involved in the pathogenesis and recovery of viral and other infectious diseases. Recombinant IFNs have been used as anti-infectious agents exhibiting a broad range of antiviral and immunomodulatory properties in both human and domestic animals. In this report, we describe a highly efficient and economical approach to purify porcine IFN alpha (PoIFNα) using polyhydroxybutyrate (PHB) as the affinity carrier and intein for self-cleaving removal of the affinity tag. Additionally, the conditions of protein expression and purification have been optimized. Our results suggested that culture medium containing 1.62% (*w*/*v*) of sodium lactate dramatically increases the accumulation of PHB binding protein in *Escherichia coli* cells. High yields of recombinant PoIFNα (30–35 mg/L, 97% purity by high-performance liquid chromatography) were obtained using intein-mediated self-cleaving conditions using a cleavage-inducing buffer with a pH of 6.5 at 20 °C for 24–36 h. The antiviral activity of the recovered recombinant PoIFNα was up to 1.4×10^6 IU/mg of protein ascertained using recombinant human IFNα1 as a standard. This report also demonstrates that large-scale production of intein-mediated purification of highly pure and active recombinant PoIFNα is feasible for the purposes of experimental studies, veterinary clinic therapeutics, and swine infectious disease control.

Keywords Intein · Poly-β-hydroxybutyrate · Porcine · IFN alpha · Purification

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Introduction

Interferons (IFNs) are a class of diffusible small molecular weight proteins induced largely in response to viral infection. The induction of endogenous IFNs occurs during the initial stage of infection, which are the first line of the host innate immune defense against infectious diseases [18]. Two types of IFNs have been identified in mammals, referred to as type I (IFNα, IFNβ, IFNω, IFNτ, and IFNλ) and type II (IFNγ). Five type I IFN subfamilies have been identified and are encoded by multigene families [24]. IFNα belongs to the type I IFN family and constitutes a cytokine family with pleiotropic functions including antiviral activity and a mediator of disease symptoms [2, 15, 18]. The antiviral activities of porcine IFNα (PoIFNα) have been widely observed in response to viral infections of the foot-and-mouth disease virus (FMDV) [6, 21, 30], porcine respiratory, and reproductive syndrome virus [4], pseudorabies virus [25], vesicular stomatitis virus (VSV) [13], transmissible gastroenteritis virus [14], and influenza viruses including the swine origin influenza A/H1N1 virus [2, 13, 22, 29]. There are numerous studies demonstrating the antiviral activity and adjuvant function of recombinant PoIFNα in various models of infection, suggesting that recombinant PoIFNα may be a potential antiviral agent that may be used to control swine virus infections such as that caused by the swine influenza virus HIN1 [7, 15–18].

Recombinant human IFNα(s) have been used clinically to enhance the ability of the host innate and acquired immune defense systems against infections for both preventative and therapeutic purposes. However, due to factors of cost and production associated with mass administration in the swine industry, using conventional purification procedures would not be feasible. Therefore, development of a simple and economical method may overcome these limitations of PoIFNα administration to the swine industry for controlling several zoonotic diseases including swine flu.

One conventional protein purification procedure used to isolate PoIFNα requires several chromatographic steps with (or without) proteolytic removal of the affinity tag. This complex procedure is costly and time-consuming resulting in extremely low yields of the product. Although using affinity chromatography to purify a tagged fusion protein may be efficient, proteolytic removal of the tag by proteases can be inefficient and leads to an increase in the cost and complexity of purification. More importantly, protease removal of the tag may inevitably contaminate the target protein. A simple and economical approach used for purification of recombinant proteins has recently been developed [1, 9]. This strategy of protein purification is based on intein-mediated self-cleaving of intein-tagged fusion proteins that were tightly bound to polyhydroxybutyrate (PHB) granules [1, 8, 9]. In this method of production and purification of target proteins, PHB granules and phasin–intein fused target proteins are expressed in *Escherichia coli* cells, where the fused target protein becomes bound to PHB granules by phasin moieties. The PHB granules can be easily separated or purified by centrifugation of cell lysate or other mechanical methods. The soluble native target protein can then be efficiently released from the granules following intein-mediated self-cleaving reaction of its C terminus by shifting the pH or temperature [1, 9].

In this report, PoIFNα was produced with high yield and purity using the PHB–intein purification system. This suggests that production of recombinant PoIFNα by intein-mediated purification approach could significantly reduce the cost of PoIFNα. Altogether, the simplicity and economics of this PHB–intein-mediated protein production and purification are promising to manufacture a large-scale and low-cost PoIFNα product that could be applied in both the prevention of swine infectious diseases and veterinary clinics.

Materials and Methods

Plasmids, Cell Lines, and Bacterial Strains

Plasmids containing the cDNA for phasin and intein (pET-PPPI:M) and PHB (pJM) were kindly provided by Dr. Wood of Princeton University [1, 9]; pET-28a was product of Novagen (USA). Plasmid pET-PPPI:M (containing a T7 RNA polymerase promoter, three copies of phasin gene and one copy of intein gene) was derived from pET-21a plasmid with Amp resistant gene as selective marker (Novagen); plasmid pJM (containing a bacterial lac promoter and one copy of PHB gene) was derived from p4A plasmid with Kan resistant gene as selective marker. Only those *E. coli* cells that harboring both pET-PPPI:M and pJM plasmids are able to grow in the medium containing Amp and Kan. Bovine VSV, Vero cells, and BHK21 cells were purchased from Shanghai Cell Bank of Chinese Academy of Sciences. Bacterial strains of *E. coli* DH5α and BLR21 (DE3) were stocks in the lab.

Reagents

Restriction endonucleases and DNA modifying enzymes were products of Takara Biologicals (Japan), New England Biolabs (USA), or Promega (USA). Chemicals used in this study were products of Sigma (USA). Plasmid mini-prep, RT-PCR, and TA Clone kits along with a DNA ladder, pre-stained protein marker, mouse monoclonal antibody against 6× His-tag, and HRP-goat anti-IgG antibodies were purchased from TianQen Biological Inc (China). Rabbit polyclonal antibody against human IFNα1 was purchased from Beijing Biosynthesis Biotechnology Co. LTD (Beijing, China). Recombinant human IFN alpha1 (HuIFNα1) standards certificated by the State Food and Drug Administration of China were obtained from the Changchun Institute of Biological Products (Changchun, China).

Porcine IFNα cDNA Cloning

Total RNA from white blood cells from a 6-month-old Bamei pig was isolated using an RNA extraction kit following the manufacturer's instructions (Takara Bio Inc, Shiga, Japan). The purified total RNA was used to generate PoIFNα cDNA using an RT-PCR kit. The primer set used for PoIFNα cDNA amplification was designed based on the published cDNA sequences of PoIFNα (GenBank: M28623, X57191, and AY76244). The primer sequences for PoIFNα cDNA are as follows: forward: 5′***TGTACA***CAACTGTGACCT GCCTCAAACCT3′ and reverse: 5′ CCC***AAGCTT***CTCCTTCTTCCTCAGTCTGT3′ with BsrG I and HindIII restriction endonuclease sites (bold and italic) of were included in the primers. The silent mutations (underlined) of CAG (the fifth codon encoding Gln) to CAA and CTC (the 161th codon encoding Leu) to CTG in the PoIFNα cDNA were also introduced to favor recombinant expression in a bacterial expression vector [10, 11]. The RT-PCR product was purified using a gel extraction kit and cloned into the PCR TA clone vector pMD18T (Takara Bio Inc, Shiga, Japan). The resulting plasmid (pMD18T-PoIFNα) was confirmed by sequencing.

Construction of Vector Expressing Phasin–Intein–PoIFNα Fusion Protein

Primers 5′CGC***CATATG***ATCCTCACCCCGGAACAAGT3′ and 5′CG***GGATCC***GTTG***TG TACA***ACAACCCCTTCGGCGACGAGGGT3′ were used for amplification of PPPI. The original pET-PPPI:M plasmid was used as the template for amplification of phasin and

intein gene by PCR [9], followed by cloning the PCR product into the pET-28a plasmid backbone resulting in plasmid pET28a-PPPI. Generation of the pET-PPPI:PoIFNα bacterial expressing vector used for PPPI:PoIFNα fusion protein expression was accomplished by ligation of the 6,067-bp fragment derived from pET28a-PPPI and the 503-bp PoIFNα fragment from pMD18T-PoIFNα (Fig. 1).

Induced Expression and Purification of the Fusion Protein

The protocol to express and purify the target proteins is illustrated in Fig. 1. The pET-PPPI:PoIFNα and pJM plasmids were co-transformed into *E. coli* BLR21 (DE3) competent cells and selected with Amp, Kan, and Tet antibiotics. Single bacterial colonies were isolated and cultured in the LB broth medium containing Amp, Kan, and Tet antibiotics (50 μg/ml for each) in a 37 °C incubator with shaking (200 rpm) overnight. The cultured bacterial cells were used as seeds to induce protein expression. One percent (*v*/*v*) of the seed culture was inoculated into LB broth medium containing antibiotics of Amp, Kan, and Tet (50 μg/ml for each) and 1.62% sodium lactate (*w*/*v*), with a 37 °C incubation for 30 h. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 1.0 mM, and the mixture was cultured for an additional 4 h. Following the IPTG induction, the bacterial cells were harvested by centrifugation, and the bacterial pellet was suspended in ten pellet

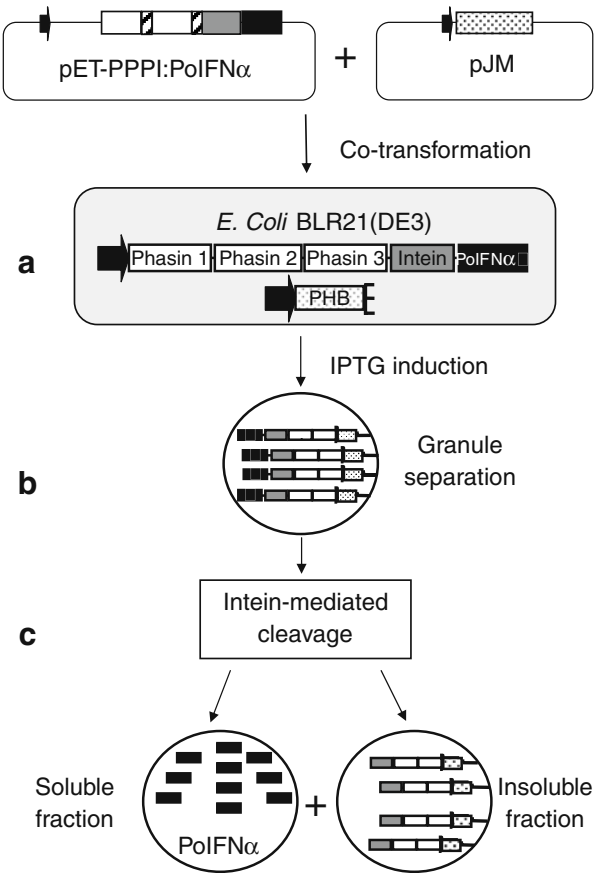

Fig. 1 Procedure of the expression and purification of recombinant PoIFNα by the PHB–intein method. The phasin–intein–PoIFNα fusion protein expressing plasmid pET-PPPI:PoIFNα and PHB expressing plasmid pJM were co-transformed into *E. coli* BLR21 (DE3) competent cells (*a*). Following IPTG-induced expression of PHB and the phasin–intein–PoIFNα fusion protein, the insoluble PHB granules with the PHB-bound phasin–intein–PoIFNα fusion protein were separated from the bacterial lysate by centrifugation and washing (*b*). The insoluble pellet was resuspended in the cleavage-inducing buffer for release of PoIFNα target protein (*c*). The insoluble PHB granules and associated protein can be removed by centrifugation leaving only the final purified PoIFNα protein in the soluble fraction [1]

volumes of lysis buffer (20 mM Tris-Bis propane, 50 mM NaCl, 1 mM DTT, 2 mM EDTA, 2.5 μg/ml lysozyme, pH 8.5). This was followed by sonication at 4 °C and the transparent bacterial lysate was centrifuged (12,000 rpm) at 4 °C for 30 min. The insoluble pellet was suspended in ten volumes of wash buffer (20 mM Tris-Bis propane, 50 mM NaCl, 1 mM DTT, 2 mM EDTA, pH 8.5) and repeated six times. The insoluble pellet was then suspended in 100 volumes of cleaving buffer (20 mM Tris-Bis propane, 50 mM NaCl, 1 mM DTT, 2 mM EDTA, pH 6.0–7.5) at 18–23 °C for 4–72 h. This was followed by centrifugation at 12,000 rpm for 30 min, and the supernatant containing target protein was harvested and sterilized using 0.45-μm pore filters, then aliquoted and lyophilized.

Determination of Protein Concentration and Purity

The protein concentration was determined by the Bradford method against known standards with slight modifications [26]. The protein expression and purity levels were ascertained by resolving the protein on a 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), followed by Coomassie G250 blue silver staining [5]. Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis was used to determine the final purity and identity of the isolated PoIFNα product as previously described [19]. Briefly, the HPLC system was balanced for 20 min before 10 μl of sample was loaded. The parameters used in the HPLC were solvent (mobile phase): methanol–1% acetic acid (85:15); stationary phase: C8 chromatography column; column temperature, 18 °C; and flow rate, 1 ml/min. The peak of protein was monitored using a UV spectrophotometer. The specificity and integrity of the purified proteins were ascertained by Western blotting using a primary mouse anti-His-tag antibody and rabbit antibody against human IFNα1. The immune complexes were detected using the DAB substrate applied on the membrane.

Determination of Recombinant PoIFNα Activity

The recombinant PoIFNα activity was ascertained by evaluation of its ability to protect BHK21 cells against bovine VSV infection in comparison to recombinant HuIFNα1 standards [20]. BHK21 cells were seeded on 96-well plates (30,000 cells per well) and cultured 4–6 h in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) before the addition of either serial diluted PoIFNα, HuIFNα1 standard, or naïve control solutions. The cells were incubated for another 18–24 h before the medium was replaced with fresh medium containing a dose of 100 tissue culture infectious dose 50 ($TCID_{50}$) of VSV to the cells [12, 27]. One interferon unit was defined as the highest dilution of IFNα that inhibited 50% of the cytopathic effect (CPE) at the time that 100% CPE was observed in naïve control cells [20].

Evaluation of Antiviral Activity of Recombinant PoIFNα by the MTT Assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to evaluate the antiviral activity of PoIFNα [3]. Vero cells ($2–3 \times 10^4$) grown at a log phase were seeded in a 96 well in 100 μl of RPMI-1640 media supplemented with 10% FBS. The medium was replaced after 8 h post-seeding with new medium containing either 100 IU of recombinant HuIFNα1, PoIFNα, or no protein control. The cells were cultured overnight before infection with bovine VSV (100 $TCID_{50}$). After an additional 24 h, 20 μl of 5 mg/ml of MTT was added to each well. Four hours later, the media was replaced with 150 μl of DMSO. The optical density (OD) at 492 nm was determined using a Microplate reader

(Model 680, Bio-Rad, Hercules, CA, USA). Each sample condition was preformed and measured in triplicate. The higher OD represented the higher IFNα antiviral activity [3].

Results

Construction of the PPPI:PoIFNα Fused Bacterial Expression Vector

Using general DNA cloning protocols and PCR technology, a fused gene of triple phasin genes (PPP), an intein gene (I), and porcine IFNα cDNA (PoIFNα) was cloned in-frame into the pET-28a bacterial expression backbone vector. The resulting construct was designated as pET-PPPI:PoIFNα, which could be used to express the PPPI:PoIFNα fusion protein following IPTG induction (for details, see "Materials and Methods" section). The resulting PPPI:PoIFNα fused gene (containing two silent mutations in the PoIFNα cDNA) was correct and in-frame as confirmed by DNA sequencing (data not shown).

Expression of the PoIFNα Target Protein

The expression of PPPI:PoIFNα recombinant fusion protein was first evaluated by transforming pET-PPPI:PoIFNα vector into the *E. coli* BLR21 (DE3) strain and incubated in LB media containing the appropriate antibiotics for 18–24 h. Following the inoculation, protein expression was induced by adding IPTG to a final concentration of 1.0 mM for 4 h. SDS-PAGE analysis of the whole cell lysate resulted in two target bands, ~83 and ~105 kDa, corresponding to the expected PPPI fusion protein (83 kDa) and PPPI:PoIFNα (105 kDa), respectively (Fig. 2a). The yield of target recombinant protein was approximately 45–55 mg when only the PPPI:PoIFNα fusion was expressed in *E. coli*. This was consistent with previous observations using a PHB–intein method to purify other proteins [1]. Further analysis of the SDS-PAGE result using Bio-Rad Quantity One gel imaging software demonstrated that the expressed fusion protein (PPPI and PPPI:PoIFNα) was about 30–40% of total bacterial protein (data not shown). These results suggested that the PPPI:PoIFNα fusion protein was successfully induced and efficiently expressed in the BLR21 (DE3) *E. coli* cells.

Purification of Porcine IFNα

The purification of recombinant PoIFNα protein was performed by following the PHB–intein method as previously described [1, 9]. As illustrated in Fig. 1, *E. coli* BLR21 (DE3) cells were double-transformed with the pET-PPPI:PoIFNα and pJM plasmids and incubated in LB media containing 1.62% sodium lactate and the appropriate antibiotics for 30 h. This was followed by 4 h of IPTG (1.0 mM) induction. SDS-PAGE results exhibited the expression of the PPPI:PoIFNα target protein with expected molecular weight of 105 kDa in the transformed cells (Fig. 2b). It was determined and calculated that approximately 45–55 mg of PoIFNα recombinant protein could be obtained from one liter of bacterial culture using the described culture conditions (Table 1 and data not shown). Following cell harvest by centrifugation, the cell pellet was lysated in lysis buffer, sonicated, and the insoluble fraction collected. This fraction was extensively washed by repeated centrifugation in washing buffer six times. The resulting pellets were PHB granules and/or granules of PHB-bound PPPI:PoIFNα fusion proteins. The SDS-PAGE results showed a single protein band with the expected molecular weight (105 kDa) of PPPI:PoIFNα fusion protein (Fig. 2c and

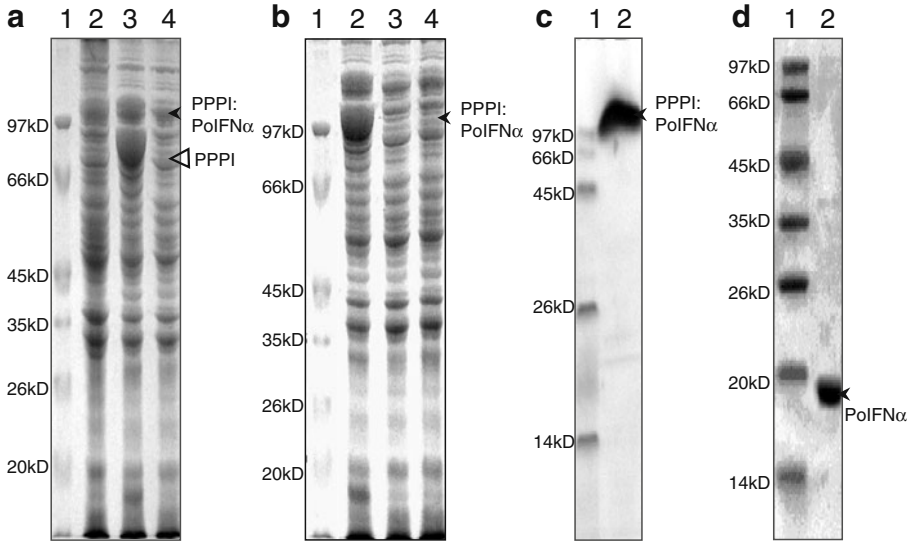

Fig. 2 Expression of the fusion protein and purification of PoIFNα. **a** SDS-PAGE results of cell lysate derived from *E. coli* BLR21 transformed with pET-PPPI:PoIFNα plasmid. *Lane 1*, molecular weight markers. *Lane 2*, BLR21 cells transformed with pJM pET-PPPI:PoIFNα plasmid but with IPTG induction. *Lane 3*, pET-PPPI:PoIFNα transformed BLR21 cells without IPTG induction. *Lane 4*, mock-transformed BLR21 control cells. Expected double phasin–intein fusion protein band (PPPI, *empty arrow head in lane 3*) and phasin–phasin–intein–PoIFNα fusion protein (PPPI:PoIFNα, *arrow head in lane 3*) were observed. **b** SDS-PAGE results of *E. coli* BLR21 co-transformed with pJM and pET-PPPI:PoIFNα plasmids. *Lane 1*, molecular weight markers. *Lane 2*, BLR21 cells co-transformed with pJM and pET-PPPI:PoIFNα plasmids but without IPTG induction. *Lane 3*, BLR21 cells co-transformed with pJM and pET-PPPI:PoIFNα plasmids and with IPTG induction. *Lane 4*, mock-transformed BLR21 control cell lysate. The expected 105-kDa band of phasin–intein–PoIFNα fusion protein band of PPPI:PoIFNα (*arrow head in lane 3*) was observed. **c** SDS-PAGE analysis showing the extensively washed insoluble fraction of cell lysate co-transformed with pJM and pET-PPPI:PoIFNα plasmids and with IPTG induction. *Lane 1*, molecular markers. *Lane 2*, insoluble fraction exhibited an expected band at 105 kDa representing the PPPI:PoIFNα fusion protein (*arrowhead*). **d** SDS-PAGE analysis displaying the soluble fraction of the post-washed PHB-bound phasin–intein–PoIFNα granules for self-cleavage of 36 h. *Lane 1*, molecular markers. *Lane 2*, soluble fraction contained the expected 19-kDa band representing the recombinant PoIFNα target protein (*arrowhead*)

data not shown). About 35–40 mg of recombinant PoIFNα could be recovered from the cell lysate from one liter of the above bacterial cultures. The recovery rate was approximately 73–78% with a purity of over 92% determined by SDS-PAGE analysis (Table 1 and data not shown). The clean insoluble pellet was then suspended in 100 pellet volumes of self-cleavage-inducing buffer (pH 6.5) to initiate the intein-mediated self-cleavage reaction at 20 °C for a time period of 4–72 h. The supernatants of cleaving reaction samples were collected and analyzed for self-cleaving efficiency and release of the target protein during the cleaving reaction. SDS-PAGE analysis indicated that PoIFNα could not be efficiently released from the granules with the incubation times of less than 20 h at 20 °C (data not shown). However, the PPPI:PoIFNα fusion protein could be efficiently cleaved to release the PoIFNα into the soluble fraction with an incubation times over 24 h at 20 °C. By SDS-PAGE, the soluble fraction resulted in a single protein band with molecular weight of 19 kDa (predicted molecular weight of PoIFNα), while the PPPI was retained in the insoluble phase with the granules (Fig. 2d and data not shown). Approximately 30–35 mg of recombinant PoIFNα could be recovered from the self-cleaving reaction using insoluble

Table 1 Yield and activity of recombinant PoIFNα purified by intein-mediated purification

Purification steps	Yield of recombinant PoIFNα/l culture			Antiviral activity (IU/mg)[b]
	Yield (mg)	Purity (%)	Recovery rate[a] (%)	
Bacterial lysate	45–55	–	–	–
Bound to PHB	35–40	>92	73–78	–
Self-cleaving	30–35	>97	64–67	1.4×10^6
Lyophilized	30–35	>97	64–67	1.4×10^6

Results from three independent experiments

[a] Compared to the indicated yields of bacterial lysate

[b] International unit per milligram of recombinant PoIFNα in comparison to the recombinant human HuIFNα1 standard

granules from one liter of the bacterial cultures. The recovery rate was approximately 64–67% with a purity of over 97% determined by HPLC (Fig. 3b; Table 1 and data not shown). The proteins in soluble fraction from the self-cleaving reaction with an incubation of 36 h was collected, sterilized, and lyophilized. The final lyophilized recombinant PoIFNα product for further analysis of the purity, integrity, identity, and activity of the purified PoIFNα. The final yield of recombinant PoIFNα was 30–35 mg from the cell lysate of one liter of the bacterial culture. The final recovery rate was about 64–67% with a purity of over 97% determined by HPLC (Fig. 3c; Table 1 and data not shown).

Purity and Specificity of the Purified Recombinant PoIFNα Protein

RP-HPLC analysis has been used to determine the purity and quality control in the products of recombinant IFNs [19]. Thus, RP-HPLC analysis was also performed to determine the purity of the final PoIFNα produced by the PHB–intein method. HPLC analysis demonstrated that a high purity of PoIFNα products could be obtained using the PHB–intein-mediated purification method. The purity was over 97% and showed a single protein peak in HPLC analysis of both the soluble fractions of self-cleaving reaction and the concentrated final product (Fig. 3a–c and data not shown). These fusion proteins were expressed using the pET vector which introduced a 6× His C-terminal fused tag. Therefore, the specificity of the fusion protein could be ascertained by detection of the His-tag and IFNα specific antibodies. Western blotting results using an anti-His-tag antibody and an anti-human IFNα1 antibody demonstrated a single specific 19-kDa protein band for the soluble fraction of the self-cleaving reaction and final protein products (Fig. 3d), indicating that the specificity of the PoIFNα proteins purified by PHB–intein method. These results demonstrate that the PHB–intein-mediated protein purification is a straightforward and economical approach to yield highly pure recombinant PoIFNα protein.

Determination of the Activity of Recombinant PoIFNα

The international unit of interferon titer is determined by evaluating the activity of interferon against VSV infection. VSV is able to replicate in cells from different species of animals and is sensitive to interferons from various species. Thus, using VSV to determine IFN activity may be a useful comparison between interferons among various species. The activity of purified PoIFNα protein was therefore evaluated by protecting BHK21 and Vero

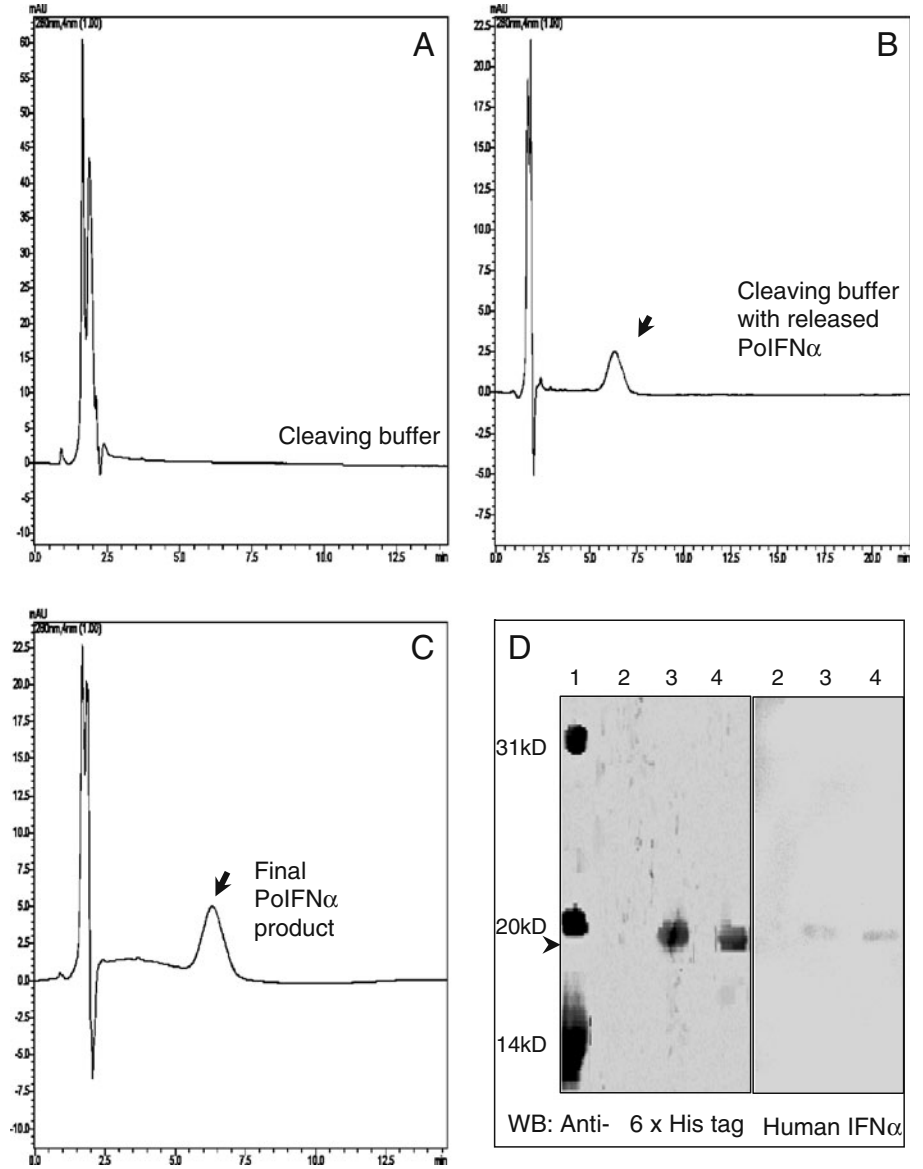

Fig. 3 The purity and integrity of the final recombinant PoIFNα. **a**–**c** HPLC analysis was employed to determine the purity and identity of the final PoIFNα products. **a** self-cleavage inducing buffer, **b** soluble fraction of self-cleaving for 36 h, and **c** the final PoIFNα lyophilized product. HPLC analysis displayed a single peak of the recombinant PoIFNα product (*arrow*) in the soluble fractions of self-cleaving reaction mixture (**b**) and final PoIFNα lyophilized product (**c**). **d** Western blot analysis of the specificity of the final PoIFNα product with antibodies against 6× His-tag (*left panel*) and human IFNα1 (*right panel*). *Lane 1*, molecular weight markers. *Lane 2*, cleaving inducing buffer used in **a**. *Lane 3*, soluble fraction of self-cleaving reaction mixture used in **b**. *Lane 4*, the final lyophilized PoIFNα product used in **c**

cells against bovine VSV infection in comparison to a recombinant HuIFNα1 which was standardized following the 1st International Reference Preparation for IFN, human leukocyte, 69/19 [20]. Recombinant PoIFNα produced by PHB–intein method demonstrated comparable antiviral activities to the HuIFNα1 standard, as seen by efficient protection of BHK21 cells against VSV infections (Fig. 4a–c). The antiviral activity of the final recombinant PoIFNα protein was 1.4×10^6 IU/mg protein, as determined by comparison to the standard (Table 1) [20]. The antiviral activity of PoIFNα was further confirmed by the MTT assay using Vero cells. Similar to that seen in the BHK21 cell protecting assay, both the recombinant PoIFNα and standard HuIFNα1 displayed effective antiviral activity to protect Vero cells against VSV infection and retain the cell proliferation following the viral infection. The PoIFNα purified by the PHB–intein method has comparable antiviral activity with the human HuIFNα standard produced by conventional

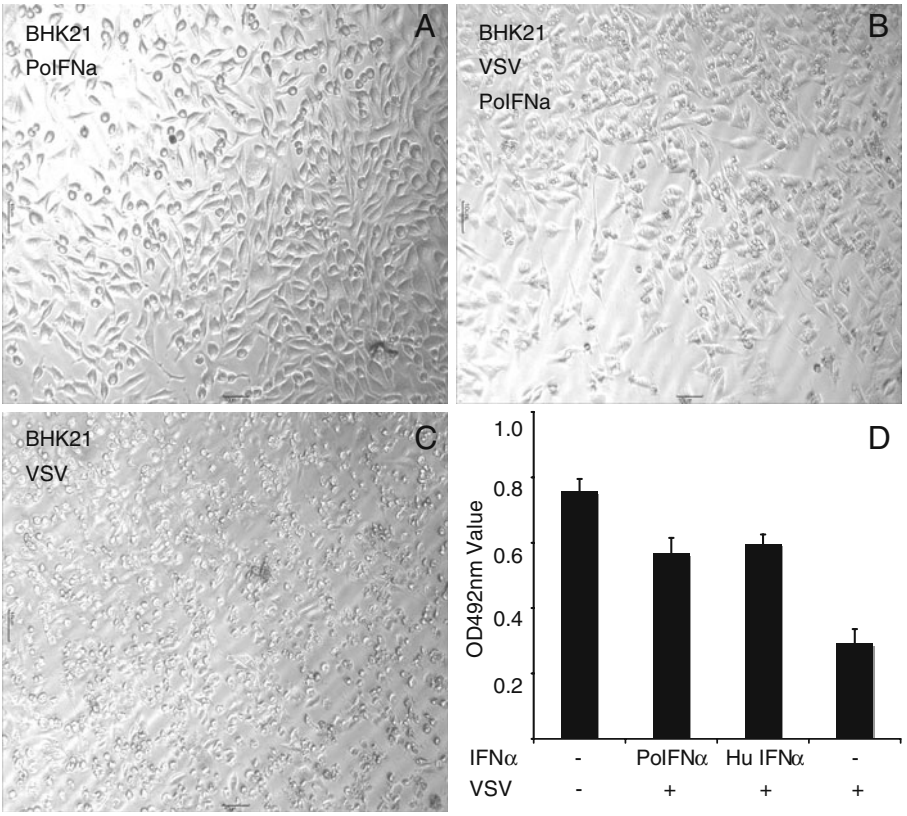

Fig. 4 In vitro antiviral activity of the recombinant PoIFNα. In vitro antiviral activity was evaluated in BHK21 cells (**a**–**c**) and Vero cells (**d**). **a**–**c** BHK21 cells were seeded in 96-well plates (30,000 cells per well) and cultured for 4–6 h before adding 100 IU of recombinant PoIFNα (**a**, **b**) or RPIM-1640 (**c**). Bovine VSV (100 TCID50) was added to the cells after 24 h following the cells exposure to PoIFNα. No CPE was observed in the cells without viral infection (**a**), antiviral activity was observed in those cells pre-incubated with PoIFNα at 12 h post-infection (**b**), and 100% CPE was observed in those cells untreated with PoIFNα at 12 h post-infection (**c**). **d** Antiviral activity detected by the MTT assay. Vero cells pre-treated with 100 IU of recombinant HuIFNα, PoIFNα, or no protein before being infected with a dose of 100 $TCID_{50}$ of bovine VSV virus

methods (Fig. 4d). These results demonstrate that the recombinant PoIFNα protein purified by the PHB–intein method retained its antiviral activity and which may be developed into a low-cost antiviral veterinary medicine in the swine industry.

Discussion

The production of highly pure and bioactive recombinant protein has been considered a time-consuming, costly, and difficult task in both the laboratory and industry settings. Although genetically introducing a protein fusion tag may simplify the purification using conventional affinity chromatography, the high expense of affinity resins and the complicated procedure of proteolytic removal of the tag may be inefficient, cause unpredicted destruction of target protein, or be contaminated with proteolytic enzymes [9, 28]. The inefficient, complicated, and costly downstream purification significantly limits the large-scale manufacture of recombinant proteins for laboratory studies, therapeutics, and other clinical applications. These limitations are a major barrier to the application of recombinant proteins in veterinary medicine used for the purposes of low-value therapeutics such as the treatment and control of animal infectious diseases. The development of recombinant protein purification strategies that are based on the combination of PHB granule–phasin affinity binding and intein-mediated self-cleaving properties are a major breakthrough in recombinant protein purification biotechnology [1, 9]. By co-expressing the intracellular polymeric granules, PHB, and phasin–intein–target fusion proteins in the same *E. coli* cells, the expressed phasin–intein–target fusion protein affinity bound to the PHB granules through phasin. This interaction was easily separated and purified by centrifugation and washing. The target protein was then released into the soluble fraction from the granule–phasin–intein–target protein complex by pH-induced intein-mediated self-cleavage leaving the PHB–phasin–intein in insoluble fraction [1, 9]. This approach has the potential to significantly simplify the procedure and decrease the expense of recombinant purification by avoiding the steps of affinity separation and/or proteolytic tag removal used in conventional purification strategies. This novel strategy could make manufacture of large-scale, highly pure, and low-cost recombinant protein possible to be used in livestock animals.

Recombinant PoIFNα has been shown to have a broad spectrum of antiviral activity against the infections of numerous viruses including the FMDV [6, 21, 30] and swine influenza virus [2, 22, 29]. The antiviral activity of recombinant porcine IFNα was 6-fold greater than the natural porcine leukocyte interferon at protection of porcine cells against virus infection [16]. However, the yield of recombinant PoIFNα produced by previous bacterial expression vectors containing the native porcine cDNA sequence was relatively low making it impracticable for large-scale preparation [16]. Previous studies have demonstrated that each gene in a genome tends to conform to its species' usage of the codon catalog; the alteration of a synonymous codon in a gene will not affect the nature of the protein but may change the expressivity of the gene [10]. In attempt to enhance the expression of PoIFNα using bacterial vector, silent mutations were generated by changes to the fifth encoding codon CAG (Gln) to CAA (Gln) and the 161th encoding codon CTC (Leu) to CTG (Leu) of the porcine IFNα cDNA sequence. These codon optimizations are favorable for bacteria and increase the usage frequencies in a bacterial expression system. These silent mutations did significantly increase the usage frequency of the codons and resulted in elevated expression of recombinant PoIFNα in the pET bacterial expression system. The expression of recombinant proteins has the potential to reach 40% of total bacterial proteins and leading to yields of 45–55 mg of recombinant PoIFNα per liter of bacterial culture (Fig. 2; Table 1 and data not shown).

The PHB–phasin–intein-mediated purification approach required the expression and synthesis of PHB granules in bacterial cells. These granules severed as the "affinity carriers" bound to PPPI:PoIFNα fusion proteins. Insufficient expression of PHB may lead excessive PPPI:PoIFNα failure to bind PHB granules and result in decreased recovery of the target proteins. In contrast, overexpression of PHB may cause PHB over accumulation thus inhibiting bacterial growth. The addition of 1.62% (*w*/*v*) of sodium lactate in the culture medium was an optimized condition for PHB expression and granule formation. The presence of sodium lactate in culture maintained the bacteria in a state of an excessive carbon source. The activity of intein is temperature and pH dependent, with the optimized temperature of 20 °C. Its self-cleaving activity decreases gradually with increasing temperature until reaching 30 °C where complete loss of cleaving activity is observed [23]. The pH value in the cleavage-inducing buffer is also critical for efficient intein-mediated self-cleavage and stability of the target protein. The intein-mediated cleavage can be initiated by shifting the pH to a weakly acidic condition, lower pH leads to a higher cleavage rate. However, the cleaving buffers with low pH increase the instability of the target proteins. A pH value over 6.5 may decline the self-cleaving rate even the cleaving may occur at pH as high as value of 7.0 [1, 9]. In this study, the intein-mediated self-cleaving reaction was performed in a buffer of pH 6.5 at 20 °C for 24–36 h. This resulted in high yields of target PoIFNα protein released into the soluble fraction of cleaving reaction, suggesting that an efficient intein-mediated self-cleaving was taken place under these conditions (Fig. 2d; Table 1).

In conclusion, we have successfully constructed a bacterial expression vector that was capable of expressing the PoIFNα fusion protein at high levels. By using the PHB–phasin–intein recombinant protein purification method, we have developed a simple and economic method to produce an experimental scale of recombinant PoIFNα protein with high purity and antiviral activity. This method may allow the manufacture of a large scale of inexpensive PoIFNα acceptable for use in therapeutics and controlling of infectious diseases in the swine industry.

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