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Expression, purification, and bioactivity of a soluble recombinant ovine interferon-tau in *Escherichia coli*

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Abstract

Introduction: Ovine interferon-tau (oIFN-τ) is a newly discovered type I interferon. This study used biochemical techniques to transform the oIFN-τ gene into *Escherichia coli* to obtain the mass and soluble expression of the recombinant protein. **Material and Methods:** First, total RNA was extracted from fresh sheep embryonic tissues with TRIzol reagent and then used as a template to reverse transcribe and amplify the mature oIFN-τ gene with RT-PCR. The amplified product was next digested with the *Hind*III and *Xho*I restriction enzymes and inserted into the pET-32a(+) vector to construct the prokaryotic expression plasmid. The corrected in-frame recombinant plasmid, pET-32a(+)-oIFN-τ, was transformed into *E. coli* Rosetta (DE3) competent cells. After induction with isopropyl-beta-D-thiogalactopyranoside (IPTG), the recombinant protein was detected in bacteria. Finally, the bacteria were lysed by sonication, and the recombinant protein was purified by nickel affinity chromatography and DEAE anion exchange chromatography. **Results:** The protein was confirmed to be oIFN-τ, which mainly existed in the soluble lysate fraction, as proven by SDS-PAGE and Western blot assays. **Conclusion:** Purified IFN-τ exists mostly in a soluble form, and its anti-vesicular stomatitis virus (VSV) activity reached 7.08×10(6)IU/mL.

Keywords: ovine interferon-tau, cytopathic effect inhibition assay, soluble expression, purification, antiviral activity.

Introduction

Interferons (IFNs) are a large protein family with antiviral, immunomodulatory, and cell growth regulatory activities (24). Isaacs and Lindenmann (5) first reported in 1957 that influenza virus-infected chicken cells could produce a soluble factor affording resistance to homologous and heterologous viruses. Currently, IFNs are generally classified into type I, type II and type III (13). Type I IFNs consist of IFN-α, IFN-β, IFN-ω, IFN-τ, and IFN-κ (14), which play important roles in suppressing virus replication and cell growth (23); type II IFNs consist of only one member, IFN-γ, which plays a key role in adaptive immune responses and is crucial for activating macrophages and natural killer (NK) cells (6); and type III IFNs consist of IFN-λ and interleukin (IL)-28/29 (20), which act antivirally similarly to type I IFNs. Although many IFNs have some overlapping effects, type I interferons have the strongest antiviral activity among the three interferons (25).

Interferon-tau (IFN-τ) is a newly discovered interferon, and its initially discovered role is to identify the signal ruminant maternal pregnancy conceptus via an anti-luteum dissolution. Subsequent studies confirmed that IFN-τ is a type I interferon and has the common characteristics of type I interferons; it has antiviral, anti-cellular proliferation, immune regulation and other functions (22). However, IFN-τ also has its own unique biological functions; it is only expressed in embryonic trophoblasts and cannot be induced by viruses (4). In addition, high concentrations of IFN-τ showed less cytotoxicity than other type I interferons (4). IFN-τ displays

a wide spectrum of antiviral activities against a variety of human and animal viruses. Its amino acid homology with IFN-α and IFN-ω is 55% and 70%, respectively, which allows it to bind to the type I interferon receptor and activate the Janus kinase-signal transducer and activator or transcription (JAK-STAT) signalling pathway, eventually leading to the generation of antiviral proteins such as PKR, ADARI, OAS, and Mx (19). Based on the characterisation of strong antiluteolysis, antiviral, immunomodulatory and other biological functions, IFN-τ has antiretroviral (*e.g.* human immunodeficiency virus) properties, and in the treatment of demyelinating multiple sclerosis (MS) it shows distinct advantages (9). In addition, because of the high specificity of retrovirus suppression and immunomodulatory activity against autoimmune diseases and the characteristics of low cytotoxicity, researchers generally believe that it has great potential as a molecular drug (17). Because of its unique biological activity and low cytotoxicity, it brings new hope for the effective treatment of many diseases.

Although ovine IFN-τ cannot be induced by viruses, has been proven to have antiviral activity against human papilloma virus (7), human immunodeficiency virus (15), feline immunodeficiency virus (15), ovine lentivirus (8), and foot-and-mouth disease virus (26). Another therapeutically beneficial characteristics is its much lower cytotoxicity at higher concentrations than IFN-α (26). However, to date, knowledge of this interferon is still far from comprehensive. To enrich ovine IFN-τ studies and further investigate its ability to treat certain diseases, the present study used conventional RT-PCR to amplify the oIFN-τ gene and construct a recombinant prokaryotic expression vector to achieve a high expression of the prokaryotic protein. The protein product was purified, and its biological activity was further examined. The results of the study provide a solid theoretical basis for further research on the biological function of oIFN-τ and its future clinical application in effective disease treatment methods.

Material and Methods

Plasmids and bacteria. Sheep embryos were purchased from a local farmer market in Hefei city, Anhui Province, China. The pMD18-T vector was purchased from TaKaRa Bioengineering Co., Ltd. (Dalian, China); pET-32a(+) was obtained from Novagen Corporation in the United States (Madison, USA); *Escherichia coli* Rosetta (DE3) and *E. coli* DH5α competent cells were purchased from Tiangen Corporation (Beijing, China); and vesicular stomatitis virus (VSV) and bovine foetal embryonic kidney (MDBK) cells were maintained and cultured in the Department of Microbiology of Anhui Medical University.

Reagents. The restriction enzymes *Hind*III and *Xho*I with 10× buffer, TaqDNA polymerase, and dNTPs were from TaKaRa Bioengineering. T4 DNA ligase, a plasmid DNA rapid extraction kit, and a DNA gel recovery reagent kit were obtained from the Promega Company (Beijing, China); other reagents were of chemical analytical grade and provided by Sinopharm Chemical Reagent Co., Ltd (Beijing, China).

Primer design. The forward P1 and reverse P2 primers were designed and synthesized by Shanghai Sangon Bioengineering Service Co., Ltd. (Shanghai, China) according to the gene sequence of the mature IFN-τ peptide. The primer sequences were as follows: 5′-ATA ***AAGCTT*** ATGGCC TTCGTG CTC-3′ as P1 (the underlined portion is the *Hind*III restriction site) and 5′-ATA ***CTCGAG*** TCAAGG TGAGTT CAG-3′ as P2 (the underlined portion is the *Xho*I restriction site).

Construction of the protein expression vector. The total RNA of sheep embryos was extracted by the TRIzol method, and cDNA was synthesized by reverse transcription using random primers. Then, the synthesized cDNA was used as a template, and the P1 and P2 primers were used to PCR amplify the oIFN-τ gene. Reaction parameters were as follows: pre-denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 40 s, and final extension at 72°C for 5 min. After the reaction was completed, 3 μL of the PCR product was electrophoresed in a 1.0% agarose gel. The amplified product and pET-32a(+) expression plasmid were digested with the *Hind*III and *Xho*I restriction enzymes, recovered and purified, and ligated with T4 DNA ligase. After culturing the positive clones and extracting the plasmid, the *Hind*III and *Xho*I restriction enzymes were used for double digestion identification and sent to Shanghai Sangon Bioengineering for sequencing. The recombinant plasmid with the correct sequence was named pET-32a-oIFN-τ.

Inducible expression of soluble roIFN-τ in *E. coli*. The recombinant prokaryotic expression plasmid pET-32a-oIFN-τ was aseptically inoculated into LB medium containing 100 μg/mL ampicillin and incubated overnight at 37°C. The next day, single colonies of the engineered bacteria were picked and inoculated in 100 mL of fresh LB medium containing ampicillin (100 μg/mL) and cultured at 37°C in an incubator under a shaking condition (200 rpm) to an OD_{600} of approximately 0.6, and IPTG was added to a final concentration of 1 mmol/L. After induction at 32°C for 8 h, the cells were centrifuged at 8 000 × g for 5 min. The bacterial pellet was collected, 4 mL of lysis buffer was added to the pellet, and the pellet was thoroughly dissolved.

Protein aggregation and solubility analysis. After centrifugation at 12,000 rpm for 20 min, cells were collected and placed on ice, and PBS (containing 1 mmol/L PMSF) was added to resuspend the cells and break the cell walls (400W ultrasonic power; 10 s ultrasound;

15 s intermittent; 20 cycles). After centrifugation at 12,000 rpm for 20 min, the supernatant was separated, and precipitation was carried out through 1.5% SDS-PAGE analysis to determine the expression and molecular weight of the protein of interest. Next, the separated proteins were electrotransferred to PVDF membranes, which were mixed with a 5% skim milk blocking solution. Then, the oIFN-τ polyclonal antibody was used as the primary antibody (1∶2000 dilution, 37°C incubation for 1 h) and HRP-labelled goat anti-rabbit antibody was used as the secondary antibody (1∶15,000 dilution, 37°C incubation for 0.5 h). The enhanced HRP-diaminobenzidine substrate kit (Boster, Wuhan, China) was used for colour development. These elements were employed in Western blot analysis.

Purification and determination of expression products. In the first step, the cell lysate was purified by affinity chromatography using Ni^{2+}-chelated Sepharose chromatography (GE Healthcare, Piscataway, NJ, USA). First, a column containing 2 ml of His binding resin was pre-equilibrated using 20 mmol/L Tris-HCl, 500 mmol/L NaCl, and 5 mmol/L imidazole. Next, the lysate sample was loaded onto the resin and then washed with a washing buffer containing 40 mm imidazole (pH 8.0). After the column was washed, the recombinant protein was eluted with elution buffer containing 300 mmol/L imidazole (pH 8.0). In the second step, a diethylaminoethyl (DEAE)-Sepharose Cl-6B anion exchange column was used for ion exchange chromatography purification according to the manufacturer's instructions. First, the eluted proteins were combined and loaded onto the column which was next washed with 17 column volumes of washing buffer. Then, the tube with the peak protein was collected and dialysed to remove salt and imidazole with buffer at 4°C. The purified protein solution was collected and centrifuged at 12,000 rpm for 15 min. The supernatant was collected, and the protein concentration was determined by the Bradford method (10) and stored at −20°C until use. In the third step, after purification, the purity of roIFN-τ was evaluated by HPLC for which were specified an XBridge BEH 300 C4 column, PBS as the mobile phase, 0.5 mL/min flow rate, 10 μL injection volume, and 30 min total run time.

Detection of antiviral activity of purified products. Using the MDBK/VSV cell assay system, the micro anti-viral inhibition method was used to determine the antiviral activity of the protein (3, 18). The test uses the highest dilution of interferon that can inhibit 50% of cytopathic effect (CPE) as 1 unit. The biological activity unit (unit activity/unit weight or volume of solution) of interferon was calculated according to the protective ability of the tested sample (at different dilutions). During the measurement, the interferon reference standard control was set up at the same time to convert the measurement results into international units (IU/mL). The titre of the interferon sample was determined by the Reed–Muench method as previously described (16).

Statistical analysis. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to analyse the data obtained from the experiment. $P<0.05$ was considered statistically significant.

Results

RT-PCR amplification and identification of the mature oIFN-τ gene. Using P1 and P2 as primers and random cDNA synthesized by reverse transcription as a template for PCR amplification, a DNA fragment with a size of approximately 520 bp was amplified, which is equal to the theoretical calculated value of the expected length of the oIFN-τ gene fragment (Fig. 1).

Digestion and sequencing identification of the pET-32a(+)-oIFN-τ recombinant plasmid. Once extraction of the recombinant plasmid was achieved, the target band appeared after digestion with *Hind*III and *Xho*I (Fig. 1C), and DNA sequencing showed that the cloned oIFN-τ gene sequence was consistent with the sequence reported in the literature (1, 21).

Identification of the IFN-τ recombinant plasmid. After the PCR-amplified IFN-τ fragment was ligated with the digested pET-32a(+) plasmid, the ligation product was transformed into *E. coli* Rosetta (DE3) competent cells; a positive clone was obtained, and the plasmid was extracted. After the recombinant plasmid was digested with *Hind*III and *Xho*I, a target fragment of approximately 520 bp was seen, and its size was consistent with the calculated theoretical value (Fig. 1). This indicates that the oIFN-τ gene was correctly inserted into the pET-32a(+) expression plasmid.

The expression of a soluble recombinant fusion protein in *E. coli* after induction. The coding sequence of the mature oIFN-τ protein was subcloned into the prokaryotic pET-32a(+) expression vector to construct the recombinant expression plasmid pET-32a-oIFN-τ. The plasmid with the correct reading frame was transformed into *E. coli* Rosetta (DE3) competent cells and cultured at 32°C after IPTG induction. The incubated samples were analysed using SDS-PAGE analysis. Gel staining showed a dominant band with a molecular weight of 35 kDa (Fig. 2) and the target protein at approximately 35 kDa in both the lane with the induced protein supernatant and the lane with the precipitated protein. The content of the target protein in the supernatant was significantly higher than that of the precipitated target protein.

Induced expression of the recombinant fusion protein and identification of the protein product. The collected bacteria were sonicated and purified by $^{+}Ni^{2+}$-chelated Sepharose column affinity chromatography. The eluted protein solution showed obvious bands at the target location. Based on the measured protein concentration after purification, the expression level of soluble recombinant protein roIFN-τ was approximately 28.45±4.65 mg/L in the bacterial solution under induction and incubation at 32°C (24).

Purification of expressed recombinant fusion protein. The sonicated soluble fraction containing the recombinant fusion protein was purified in two steps using nickel affinity chromatography and DEAE anion exchange chromatography. Unique prominent protein peaks were found on the Ni^{2+} affinity (Fig. 3A) and DEAE (Fig. 3B) chromatograms. HPLC analysis showed the main protein peak to be in the final purified product, and that product to be at a purity of 97.83% (Fig. 4).

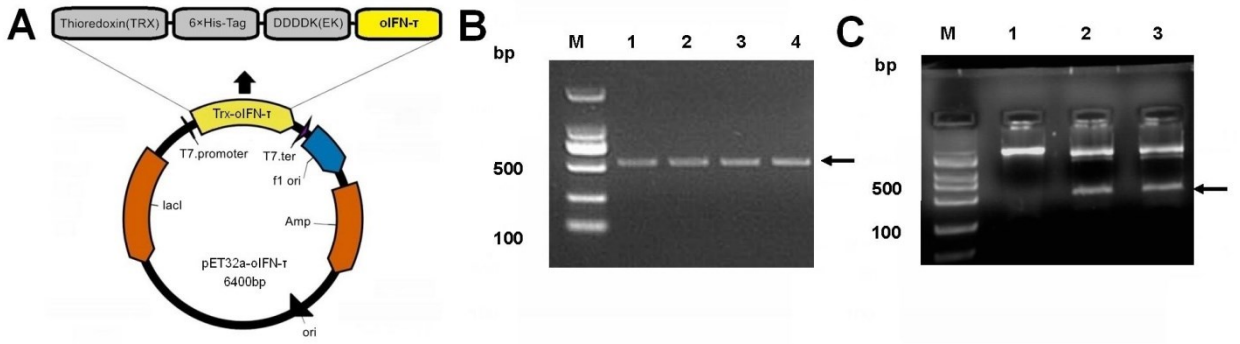

Fig. 1. Construction and identification of the prokaryotic expression plasmid (pET-32a-roIFN-τ)
A – Construction of a recombinant prokaryotic expression vector (pET-32a-roIFN-τ)
B – RT-PCR amplification profile of the roIFN-τ gene. Lane M – marker (DNA molecular weight standard); lanes 1–4 – RT-PCR product amplified from total ovine mRNA
C – Restriction map of the recombinant pET-32a-roIFN-τ expression plasmid. Lane M – marker (DNA molecular weight standard); lane 1 – single *Xho*I restriction enzyme digestion of the pET-32a-roIFN-τ recombinant plasmid; lanes 2–3 – digestion of the pET-32a-roIFN-τ plasmid using the *Xho*I and *Hind*III restriction enzymes

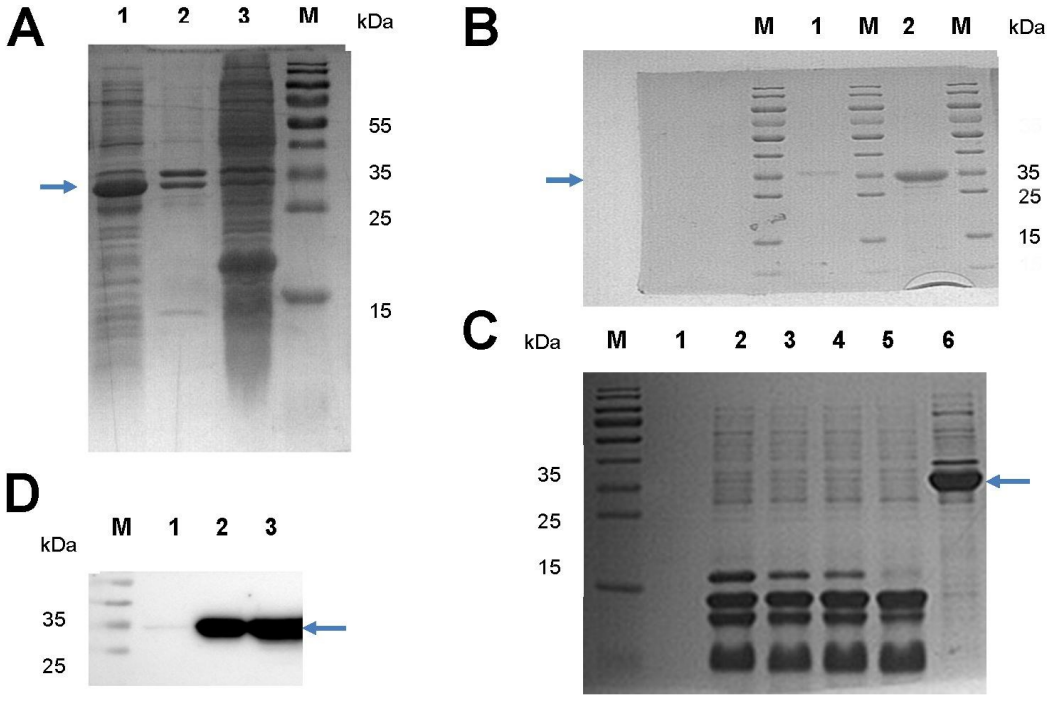

Fig. 2. The identification of the recombinant Trx-roIFN-τ fusion protein expression products by SDS-PAGE and Western blot analyses
A – Expression of the recombinant fusion protein. Lane M – protein molecular weight marker; lane 1 – soluble fraction of the bacterial lysate after sonication; lane 2 – insoluble fraction of the bacterial lysate after sonication; lane 3 – total cellular protein lysate from *E. coli* Rosetta (DE3) without isopropyl-beta-D-thiogalactopyranoside induction
B – Purification of the recombinant fusion protein. Lane M – protein molecular weight marker; lane 1 – negative control; lane 2 – recombinant protein after two-step chromatography purification
C – Identification of the Trx-roIFN-τ recombinant fusion protein. Lane M – molecular weight marker; lane 1 – negative control; lanes 2–5 – purified roIFN-τ after enterokinase digestion at different time points; lane 6 – purified roIFN-τ protein without enterokinase digestion
D – Western blot analysis of the purified protein. Lane M – molecular weight marker; lanes 1–3 – total cellular protein lysate from *E. coli* Rosetta (DE3) after induction with IPTG at 2 h, 6 h, and 10 h
Blue arrow – dominant 35 kDa band

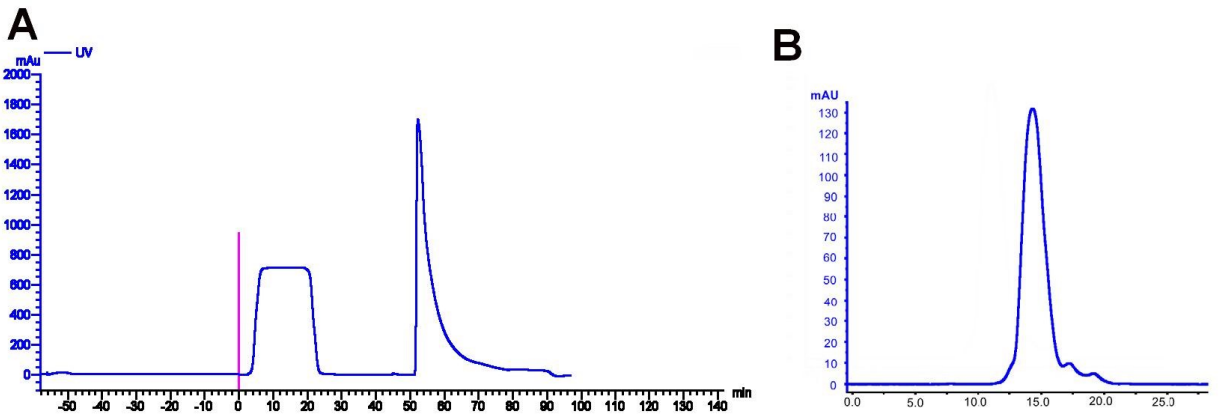

Fig. 3. The purification profiles of the recombinant protein using affinity chromatography and diethylaminoethyl (DEAE) anion exchange chromatography
A – Ni^{2+} affinity chromatography at an absorbance of 280 nm ($A_{280\ nm}$); B – DEAE anion exchange chromatography at an absorbance of 280 nm ($A_{280\ nm}$)
The abscissa (X axis) represents time with the unit of minutes (min). The ordinate (Y axis) represents the electrical signal with the unit of mAu (milli-absorbance units). The –UV symbol represents the detected purple ultraviolet curve profile

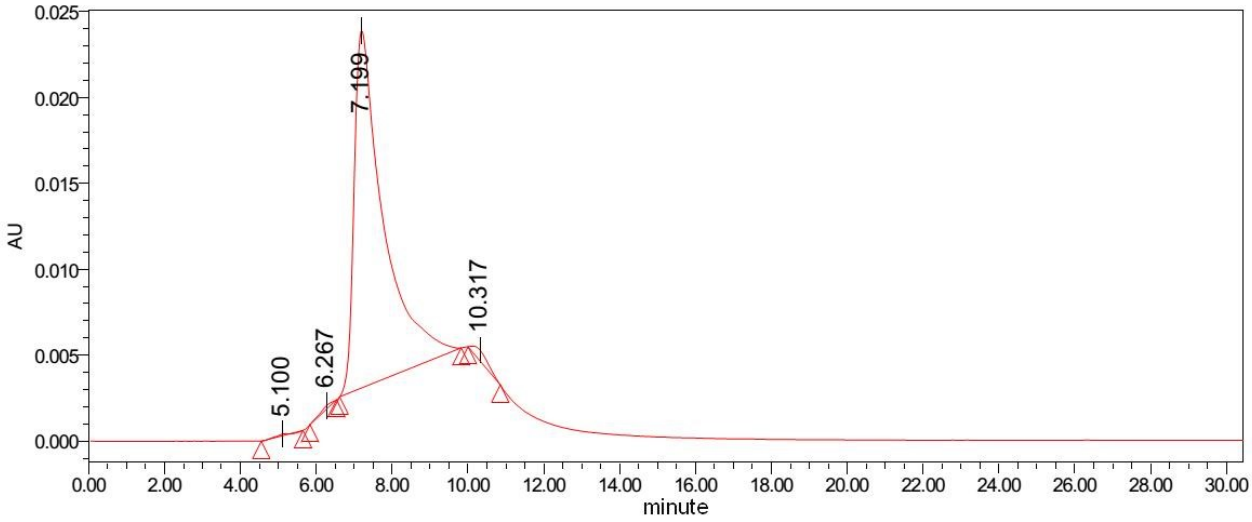

Fig. 4. Purity characterisation of the recombinant fusion protein as determined by high-performance liquid chromatography (HPLC)
Automatic scaling of HPLC chromatography was applied to measure the purity of the purified target fusion protein at a wavelength of 280 nm-AU – absorbance unit

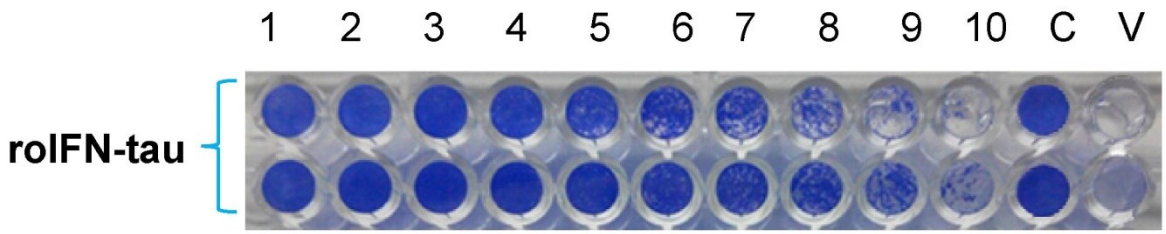

Fig. 5. Antiviral activity of the recombinant Trx-roIFN-τ fusion protein detected in the MDBK/VSV titration system
A cytopathic effect (CPE) inhibition bioassay with crystal violet staining was performed to detect the antiviral activity of the Trx-roIFN-τ fusion protein, and the results showed 50% CPE inhibition with 1 unit of roIFN-τ and the control groups for the titration of the biological activity of roIFN-τ. Column V – virus control; column C – cell control; columns 1–10 – interferon test sample serially diluted 4-fold from left to right

Table 1 Titration results of a recent batch of expressed recombinant ovine interferon-tau

Column	Dilution	Cytopathic effects record (+ or −)*	Number of protections	Number of lesions	Grand total		Protection ratio	Protection percentage
					Cumulative number of protections	Cumulative number of lesions		
1	1:4	−,−	8	0	58	0	58/58	100%
2	1:16	−,−	8	0	50	0	50/50	100%
3	1:64	−,−	8	0	42	0	42/42	100%
4	1:256	−,−	8	0	34	0	34/34	100%
5	1:1024	+,−	7	1	26	1	26/27	96.3%
6	1:4096	+,+	6	2	19	3	19/22	86.3%
7	1:16384	+,+	6	2	13	5	13/18	72.2%
8	1:65536	++,+	5	3	7	8	7/15	46.7%
9	1:262144	+++,+++	2	6	2	14	2/16	12.5%
10	1:1048576	++++,++++	0	8	0	22	0/22	0%

Identification of the expressed recombinant fusion protein. Western blot analysis was performed with the anti-roIFN-τ polyclonal antibody. The Trx tag, His tag and other protein indicators were not digested before the protein itself was, with enterokinase. The molecular weight of the protein product was approximately 35 kDa (Fig. 2). The molecular weight of the protein product after enterokinase digestion was approximately 20 kDa, which is consistent with the molecular weight of the mature roIFN-τ protein without the signal peptide (Fig. 2). These results were in agreement with the theoretical calculated molecular weights of the proteins (Fig. 1), confirming that the expressed recombinant protein products contained two fragments, Trx and roIFN-τ. The roIFN-τ protein, both before and after digestion, can react specifically with the roIFN-τ polyclonal antibody, as shown in Western blot analysis. These results indirectly prove that the expressed recombinant protein is roIFN-τ.

Activity determination of the recombinant roIFN-τ protein. As shown in Fig. 5, the purified roIFN-τ protein effectively inhibited the cytopathic effect of VSV in MDBK cells, as shown by its obvious anti-VSV activity. After calculation, the dilution rate of the IFN-τ sample corresponding to the percent inhibition of cytopathic effects higher than 50% was $1:10^5$, and the IFN-τ titre was $10^{5.85}$ IU/100 μL = $10^{6.85}$ IU/mL = 7.08×10^6 IU/mL, as shown in Table 1.

Discussion

Interferons (IFNs) are a large class of functional proteins that can be generated after a virus infects the body. They have a wide range of antiviral, antitumour and immunoregulatory effects. In the interferon protein family, there are also type II and type III interferons in addition to type I. As a new discovery in this last category, IFN-τ has its own characteristics besides those common to all type I interferons, with anti-luteal lysis, nonviral induction, cross-species activity, and nontoxicity or low-toxicity features (11). Nontoxic side effects make it a molecular drug with more advantages than the older IFNα/β proteins and bring new hope for the treatment of many diseases (1). Therefore, IFN-τ has wide clinical application value and huge market prospects. Currently in the United States, recombinant oIFN-τ has entered phase III clinical trials for the treatment of human multiple sclerosis, psoriasis, rheumatoid arthritis and hepatitis. The acquisition of pure IFN-τ is a crucial prerequisite for exploiting its therapeutic potential. However, IFN-τ is only expressed at a specific time and in a specific location (*i.e.*, it is secreted by trophoblast cells during a period before and after implantation), and the direct tissue culture and extraction processes are complicated, fraught with difficulties and hampered by limitations.

The CPE inhibition assay is a common method used to determine the antiviral activity of an interferon. Interferons can stimulate certain indicator cells (such as human amniotic epithelial cells of the WISH strain, human laryngeal cancer cells of the Hep-2 strain, and human embryonic muscular skin or lung monolayer cells) to produce antiviral proteins, thereby protecting the cells from, for example, vesicular stomatitis virus, a favoured challenge virus for the assay (2). Such protection is called cytopathic effect inhibition.

Expressing recombinant IFN-τ through genetic engineering technology has become an effective method. The current foreign gene production method is primarily the *E. coli* prokaryotic and yeast eukaryotic expression system (12). An IFN-τ *Saccharomyces cerevisiae* eukaryotic system has been constructed in which the soluble expression of IFN-τ was achieved, and the antiviral activity reached 6.0×10^6 U/mg equalling the natural IFN-τ activity, but the expression system could not be scaled up (12). To expand IFN-τ production, Van Heeke *et al*. (27) and Sinha *et al*. (21) enhanced the fermentation yield of IFN-τ using *Pichia pastoris* as the expression host and obtained 280 mg/L and 391.7 mg/L concentrations, respectively. The yeast eukaryotic expression system can be used even when IFN-τ is expressed in its active form, but the production process is time consuming and costly because of the low yield.

Our group selected the prokaryotic *E. coli* expression system due to its clear genetic background, easy cultivation, and the availability of a large number of prokaryotic expression vectors (e.g. pET series vectors) for exogenous protein production, leaving us a choice of different ones for the soluble expression of oIFN-τ. In this study, we used the pET-32a(+) vector, which contains the Trx solubility cofactor, successfully constructed the IFN-τ prokaryotic expression system of pET-32a(+)-oIFN-τ, and achieved a high expression of oIFN-τ. The oIFN-τ products are in an active and suitably soluble form, with a relatively simple purification process for >95% purity, moderate yield, and high antiviral activity, which all recommend that this expression system can be used in further research applications and the protein in future clinical applications. Therefore, there is hope that the present study will contribute to the future application of roIFN-τ and the treatment of ovine viral diseases.

* These authors contributed equally to this study and should be considered co-first authors.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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