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Short communication

Intein-mediated expression and purification of common carp IFN-γ and its protective effect against spring viremia of carp virus

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ABSTRACT

IFN-γ is a pleiotropic cytokine with significant roles in antiviral, antitumor and immune regulation. It could be used as an immuno-enhancer to improve fish protectiveness against pathogens. In this study, the prokaryotic expression plasmid pTwin1-N-IFN-γ was constructed to express *Cyprinus carpio* (common carp) IFN-γ fused with a chitin binding domain (CBD) and a self-cleavable intein-tag, *Synechocystis sp* DnaB. The recombinant protein CBD-DnaB-IFN-γ with the molecular weight of 44.25 kD was successfully expressed in soluble form, and the rIFN-γ (approximate 18.61 kD) was further cleaved and eluted under pH = 7.0 at 25 °C. rIFN-γ could be recognized by western blotting with rabbit anti-grass carp IFN-γ polyclonal antibody. Cytotoxicity studies on EPC cells showed that only 500 ng/ml rIFN-γ had a subtle effect on cells growth and its proliferation rate was reduced to 76.2%. EPC cells incubated with 100 ng/ml rIFN-γ showed significantly higher resistance against SVCV, reducing the $TCID_{50}$/ml by more than 800-fold. In vivo studies suggested that intraperitoneal injection of rIFN-γ significantly improved the survival rate of common carps compared with SVCV challenge alone. These results implied that rIFN-γ would act as an immuno-enhancer in carp aquaculture.

1. Introduction

Cyprinus carpio (common carp) is one of the important freshwater-farming fish species in China with the annual mount of over 3, 000, 000 ton [1]. Its farming was threated seriously by bacteria, fungi and virus especially Spring viraemia of carp virus (SVCV) [2]. SVCV, which was highly lethal to young fish with over 90% mortality, was firstly detected in Yugoslavia in 1971 [3], and has been reported in Europe, America and several Asian countries. SVCV could naturally or experimentally infect cyprinid fish as well as other non-cyprinid fish such as salmon, catfish etc and caused significant morbidity and motality [4,5]. Once the ponds suffered from SVCV invasion, the result of eradicating the virus was destructive for most aquatic life [6,7]. Due to the geographical distribution and wide host range, SVCV became a threat not only to the seafood and ornamental fish trade industries, but also to local wild fish populations, and successfully attracted attention of China's ministry of Agriculture and the world organization for animal health [4,5].

Type II interferon is a pleiotropic cytokine discovered in vertebrates with significant roles in antiviral, antitumor and immune regulation [8,9]. In tetrapods, there is only one member of type II interferon, IFN-γ. In some piscine such as channel catfish *Ictalurus punctatus*, goldfish *Carassius aurutus* L., grass carp *Ctenopharyngodon Idella* etc, another member of IFN-γ, IFN-γ related gene (IFN-γ-rel), was also found [10–12]. The IFN-γ sequences share conserved signaling peptide, IFN-γ signature motif and nuclear localization sequence (NLS), while NLS motif is lacked in IFN-γ related gene [13]. Stolte et al. reported that two IFN-γ genes in common carp exhibit distinct functions, such as IFN-γ-2 (resembling to other vertebrates IFN-γ gene) referring to T-lymphocyte response, and IFN-γ-1(IFN-γrel) referring to B lymphocyte response [14]. In 2010, Arts et al. also proved that IFN-γ-2 but not IFN-γ-1 induces the classical activation of phagocytes, thus triggering a TH-1 like profile immune activation [15]. However, its antiviral function has not been studied. In the present study, recombinant IFN-γ was expressed and purified, and its protective effect against SVCV was studied *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cells, virus and fish

Epithelioma papulosum cyprinid (EPC) cells were cultured in Medium 199 (Hyclone) containing with 10% fetal bovine serum (FBS, Biological Industries), 100 IU/ml penicillin and 100 μg/ml streptomycin (Biological Industries) at 25 °C for passage. SVCV was kindly provided by Professor Nie in Institute of Hydrobiology, Chinese

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Academy of Science and propagated in EPC cells. Common carps with the body length of 2.5 ± 0.5 cm were raised at 20 ± 1 °C in plastic tank for two-weeks domestication and fed with commercial puffed fish compound particle feed (156# with 3–4 mm diameter, Tongwei Co. LTD) twice a day.

2.2. Recombinant plasmid construction

Plasmid pTwin1 is a vector which utilizes the specific self-cleavage of inteins to separate the target protein from the affinity tag. There are three strategies for subcloning, fusing the intein on the N-terminus, C-terminus or both ends of target protein. In this paper, the N-terminal fusion strategy was employed as manufactures' illustration. Firstly, a pair of primers IFN-γ*Nco*IF(5′-TGCCATGGGCAGCGTCCCTGAGAAC CTG-3′)/IFN-γ*Pst*IR(5′-AACTGCAGTTAAGACTTTTGCTTT-3′) was designed to amplify the mature peptide of IFN-γ based on the mRNA sequence of common carp *IFN*-γ in GenBank (JX181980.1). The amplified PCR fragment was then double-digested with restriction enzymes of *Nco*I and *Pst*I, and ligated into linearized pTwin1 plasmid with the same enzymes, before being transformed into *Escherichia coli* (DH5α). The positive clone was verified by sequencing and recombinant plasmid pTwin1-N-IFN-γ was extracted, before being transformed into an *E.coli* BL21 expression strain.

2.3. Recombinant IFN-γ expression and purification

The N-terminal fusion strategy utilized the self-cleaving activity of *Synechocystis sp* dnaB under pH = 7.0 at 25 °C condition [16,17]. Therefore, the purification of the recombinant IFN-γ (rIFN-γ) was very convenient just for changing different pH circumstance following the manufactures' instruction. Briefly, the freshly grown culture of *E.coli* BL21 transformed with pTwin1-N-IFN-γ were cultured at 37 °C and 200 rpm in LB medium with 100 μg/ml final concentration ampicillin. When the OD_{600} reached 0.5, cultures were induced at 18 °C overnight with 0.1 mM IPTG for the inductive expression of the fusion protein CBD-DnaB-IFN-γ. Cells were harvested by centrifugation and re-suspended in ice-cold Buffer B1 (20 mM Tris-HCl, 500 mM NaCl, pH = 8.0) before sonication. The supernatant of sonication was added to a chitin column equilibrated with Buffer B1 for binding through CBD tag. Column was washed with Buffer B1 to remove the unbound proteins, before equilibrated the chitin resin in Buffer B2 (20 mM Tris-HCl, 500 mM NaCl, pH = 7.0) at 25 °C overnight for on-column cleavage of the intein-tag DnaB. Finally, the target protein IFN-γ was eluted with Buffer B2 and replaced with PBS (pH = 7.4) by ultrafiltration with Amicon ultra filter (10 kD molecular mass cutoff), before being loaded onto a polymyxin B column (Sigma) to reduce the potential bacteria endotoxins contamination. The Bradford method was used to measure the concentration of rIFN-γ. Meanwhiles, the rIFN-γ was determined by western blotting with rabbit anti-grass carp IFN-γ polyclonal antibody prepared in Professor Nie's lab before.

2.4. Cell proliferation analysis

EPC cells were cultured separately in 96-well plates at the density of 5×10^3 cells/well and cultured for 24 h rIFN-γ at final concentration of 0, 15.6, 31.3, 62.5, 125, 250, 500 ng/ml was added into each well for another 48 h incubation. Meanwhiles, cells treated with different concentrations of cis-Diammineplatinum dichloride (CAS:15663-27-1) were used as positive control. Cell proliferation was determined using cell-counting kit-8 (Beyotime) following the manufactures' protocol. The optical density was measured at 450 nm with a reference wavelength of 630 nm, and cells proliferation without any treatment was thought as 100%. Each treatment was run in six replicates. Independent samples T-test was used to analyze the significant differences (*, $P < 0.05$) between the control and experiment groups.

2.5. Antiviral activity analysis

The antiviral activities of rIFN-γ were determined by the resistance against SVCV *in vitro* and *in vivo*. In vitro, EPC cells were cultured separately in 96-well plates at the density of 5×10^4 cells/well and cultured for 24 h. Cells were treated with control or 100 ng/ml rIFN-γ for another 24 h, before being infected with 10-fold series dilutions of SVCV. Virus titers were calculated till no CPE appeared according to the method reported by Reed and Muench [18]. After infection, cells were fixed with formaldehyde and stained with 0.05% crystal violet solution.

Common carps (n = 180) were divided randomly into six groups. Carps from three groups were intraperitoneally injected with 50 μl 6.0×10^7 $TCID_{50}$/ml of SVCV in PBS (referring to Ref. [19]) as control and carps from the other three groups were intraperitoneally injected with 50 μl 6.0×10^7 $TCID_{50}$/ml of SVCV along with 1000 ng rIFN-γ in PBS as experiment groups. The relative percentage survival was recorded daily for 20 days. Independent samples *t*-test was used to analyze the significant differences (*, $P < 0.05$) between the control and experiment groups.

2.6. Ethics statement

The Animal Ethic Committee of Jiangxi Normal University approved all experimental procedures used in the present study.

3. Results

3.1. Recombinant protein purification

As shown in Fig. 1, the fusion protein CBD-DnaB-IFN-γ with the predicted molecular weight of 44.25 kD was shown in DE3 cells transformed with pTwin1-N-IFN-γ with IPTG induction. After on-

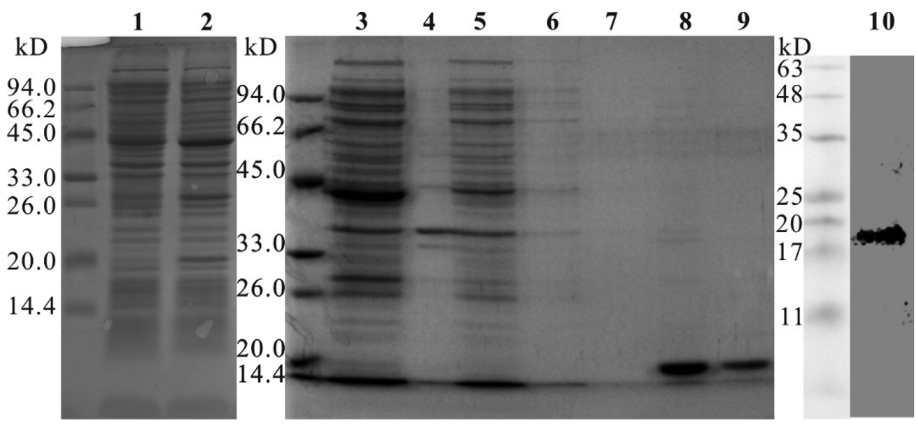

Fig. 1. The purification of rIFN-γ. Lane 1,DE3 transformed with pTwin1-N-IFN-γ without IPTG induction; Lane 2, DE3 transformed with pTwin1-N-IFN-γ with 0.1 mM IPTG induction; Lane 3/4, The sonication supernatant/pellet of DE3 transformed with pTwin1-N-IFN-γ with 0.1 mM IPTG induction; Lane 5 The flow-through fraction after sonication supernatant being loaded on column; Lane 6/7, the flow-through fraction of column washed with Buffer B1/B2; Lane 8/9, the elution fractions of column washed with Buffer B2 after self-cleavage; Lane 10, western blotting detection of rIFN-γ.

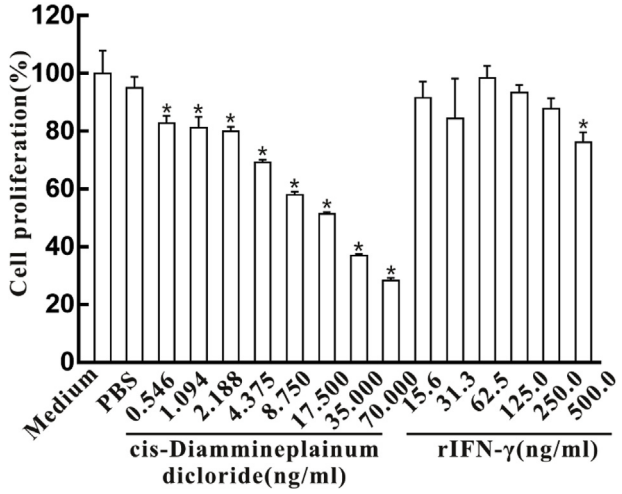

Fig. 2. The effect of rIFN-γ on EPC cells growth. EPC cells were incubated with different concentrations of rIFN-γ or cis-Diammineplatinum dichloride for 48 h. Cells proliferation rate was determined by CCK-8 kit. Each treatment was run in six replicates. Independent samples *t*-test was used to analyze the significant differences (*, $P < 0.05$) between the control and experiment groups.

column cleavage of the intein-tag DnaB, rIFN-γ with the predicted molecular weight of 18.61 kD was eluted. The western blotting result showed that the rIFN-γ was recognized by a rabbit anti-grass carp IFN-γ polyclonal antibody.

3.2. Effect of rIFN-γ on EPC cells growth

The cytotoxicity of rIFN-γ in EPC cells was determined by incubation with various concentrations of rIFN-γ (Fig. 2). cis-Diammineplatinum dichloride, an agent could inhibit tumor cells growth and promote apoptosis [20] was used as positive control and reduce the EPC cells proliferation to 28.3% at concentration of 0.07 mg/ml. The rIFN-γ concentration ranges from 15.6 to 250 ng/ml had no significant effects on EPC cell proliferation, while rIFN-γ at the concentration of 500 ng/ml had a subtle effect on EPC cells growth and its proliferation rate was reduced to 76.2%.

3.3. Effect of rIFN-γ on EPC cells resistance against SVCV

The crystal violet staining result showed that EPC cells infected with 10^8-fold diluted SVCV stock kept intact in control group, while EPC cells infected with over 10^5-fold diluted SVCV stock kept intact after treatment with rIFN-γ (Fig. 3A). The $TCID_{50}$ of cells incubated without rIFN-γ was $10^{-7.55}$/0.1 ml, while $TCID_{50}$ of cells incubated with rIFN-γ was $10^{-4.65}$/0.1 ml (Fig. 3B). The relative percentage survival was also analyzed *in vivo*. Common carps were intraperitoneally injected with rIFN-γ along with SVCV showed a significant higher survival rate (47.7%) compared with control group (0%) (Fig. 3C).

4. Discussion

Prokaryotic expression system was often used to obtain exogenous protein for function analysis. Exogenous protein with tags could be easily purified, while recombinant proteins without tags are crucial to maintain its natural state. Art et al. firstly failed to express rIFN-γ-2 using pQE30 expression vector, but successfully obtain the soluble rIFN-γ-2 with pET15 expression vector after codon optimization of rIFN-γ-2 gene for bacteria expression [15]. The recombinant protein present in the insoluble fraction could be denatured and then refolded in refolding solution. However, when the refolded protein showed no activity, it was controversial that the reason was due to its character or unproper refolding. In this study, pTwin1 vector using intein mediated on-column self-cleavage purification system was used to obtain common carp rIFN-γ. Although the rIFN-γ was expressed with a CBD tag and an intein tag DnaB (up to 25 kDa), the rIFN-γ was cut off and the tag was left on-column under pH = 7.0 at 25 °C without any additional reductive agent or protease. The intein-mediated purification was especially suitable for peptide preparation as small as 2-3kD such as antimicrobial peptide BR2, β-defensin etc [21,22].

Antiproliferative effect is one of the important roles of IFN-γ. IFN-γ could up-regulate PKR, p21, p27, Rb etc expression and down-regulate c-myc expression to inhibit cellular proliferation [8,9]. In the present study, rIFN-γ at high dose of 500 ng/ml had a slight inhibition on EPC cells proliferation (Fig. 2). Another important role of IFN-γ is the ability of antiviral. IFN-γ could induce PKR, ADAR, GBP1, GBP2 etc expression to resist viral propagation [8,9]. Compared with type I interferon, IFN-γ builds a later antiviral state for longer term control dependent on type I interferon signaling pathway [23–25]. In ZF4 cell line, IFN-γ could protect against the SVCV as well as type I IFNs [26]. IFN-γ in tetrapods such as Chinese soft-shelled turtle, goose, mouse etc also showed antiviral effects against its species-specific virus replication *in vitro* [27–29]. In this study, rIFN-γ increased EPC cells resistance to 800-fold against SVCV compared to control treatment (Fig. 3B). Since SVCV caused serious mortality to carp, researchers attempt to develop an effectual vaccine to control SVCV. Based on the SVCV glycoprotein (G) gene, Kanellos etc al designed ten different DNA vaccines and investigated their protecting ability against SVCV [30]. The result showed that fish immunized with expressing full length G-gene plasmids driven by the CMV-Intron A promoter possessed the highest percentage survival of 48%. Another DNA vaccine expressing the G gene from North American SVCV isolate exhibited a strong protective response against SVCV in ornamental koi and goldfish with 50–85% survival rate [31]. Besides DNA vaccines, oral vaccines referring to G protein was also investigated. The oral vaccine *Lactobacillus plantarum* co-expressing SVCV G protein and koi herpesvirus ORF81 protein showed 71% and 53% protection rates for carps and koi respectively [32]. In this study,

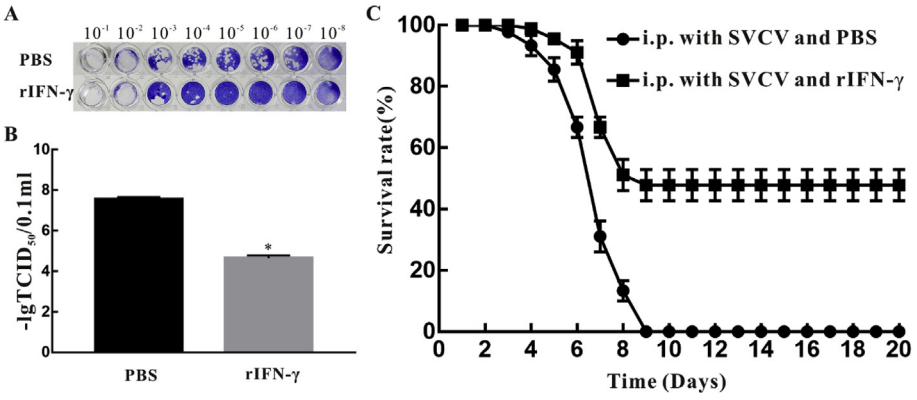

Fig. 3. The antiviral ability of rIFN-γ. A, The crystal staining of EPC cells infected with 10-fold diluted SVCV stocks; B, The effect of rIFN-γ on SVCV TCID50 in EPC cells, independent samples *t*-test was used to analyze the significant differences (*, $P < 0.05$) between the control and experiment groups; C, The effect of rIFN-γ on survival rate of carps against SVCV.

common carps intraperitoneally injected with rIFN-γ along with SVCV showed an increased protective effect with 47.7% survival rate (Fig. 3C), indicating that IFN-γ could act as an immuno-enhancer in carps aquaculture. IFN-γ was also used as an immunoadjuvant to enhance specific antibody and specifically protect against viral infections in other mammals such as weaning piglets and mice [33,34].

In conclusion, common carp rIFN-γ was successfully expressed and purified using intein-purification system and exhibited activity of resisting SVCV infection both *in vitro* and *in vivo*, providing a potential immuno-enhancer in carps aquaculture.

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