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Full Length Article

Expression, Purification and Characterization of Recombinant Interferon-γ of *Takifugu rubripes* in *Pichia pastoris*

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Abstract

Interferon gamma (IFN-γ), a major cytokine, has broad-spectrum antiviral function, in addition to play a key role in the process of immune regulation. In this study, in order to explore the biological roles of recombinant IFN-γ (rIFN-γ), IFN-γ was cloned from *Takifugu rubripes* and expressed in *Pichia pastoris* under the control of an electrical process. *P. pastoris* were screened by high concentration culture medium of Zeocin, producing the high-copy number *P. pastoris* induced by culture medium of BMGY/BMMY. We used mass spectrometry (MS) analysis to identify the rIFN-γ protein. The results showed that the molecular weight of rIFN-γ was 24 kDa by SDS-PAGE gel electrophoresis, the bioactivity of rIFN-γ was 2018 U/mL and the concentration of the rIFN-γ protein was 0.242 mg/mL after inducing for 96 h. Also, we identified the rIFN-γ protein of *T. rubripes.* rIFN-γ could stimulate WISH cell proliferation, which was associated with recombinant protein concentration. These results provide a basis for application of the rIFN-γ protein as a vaccine candidate against pathogen infection in aquaculture. © 2019 Friends Science Publishers

Keywords: *Takifugu rubripes*; Interferon gamma; Characterization; Eukaryotic expression; *Pichia pastoris*

Introduction

The cytokine interferons (IFNs) influence the regulation of the immune response (Samuel, 2001; Kong *et al*., 2018). The mammal IFNs (types I to III) have been well characterized and can be quickly produced in response to pathogen infection. *IFN*-γ is the natural glycoprotein and primarily produced by T helper and natural killer cells. IFN-γ plays important roles in antiviral activity, antigen presentation and processing. *IFN*-γ also can enhance the cytotoxicity and cell-mediated immune responses of producers (Collins *et al*., 1984; Schroder *et al*., 2004; Miller *et al*., 2010). Recently, IFN-γ has been used in a wide variety of clinical indications, such as cancer, tuberculosis, mycobacterium avium complex (MAC) and idiopathic pulmonary fibrosis (Miller *et al*., 2010). Additionally, IFN-γ has antiviral activity in mammals (Zamani *et al.*, 2007; Imran *et al.*, 2014). Many studies have proven that IFN-γ may have therapeutic value for musculoskeletal diseases in veterinary medicine and can be an adjunct tool in contact investigations of latent tuberculosis infection in healthcare workers (Jonnalagadda *et al*., 2013; Tsang *et al.*, 2015; Maumus *et al*., 2016). However, research on fish IFN-γ started relatively late compared to research in mammals.

Teleost fish have at least two types of *IFN* genes. In teleost fish, type II IFNs include two members, which are termed IFN-γ and IFN-γ-related molecules (Zou *et al*., 2005; Chen *et al*., 2010; Swain *et al*., 2015). In fish species, the IFN-γ gene has also been identified and characterized, whose gene structure was identical with mammalian *IFN-γ* genes (Robertsen, 2006). The syntonic IFN-γ loci were also conserved in the genome of all vertebrates, indicating that the basic functions of the vertebrate IFN-γ gene may share a common evolutionary origin (Savan *et al*., 2009). According to higher homology in the genomes of rainbow trout and Atlantic salmon, the rainbow trout *IFN-γ* gene was screened from the cDNA library of its head kidney cells. Additionally, a comprehensive analysis of two *IFN-γ* genes in channel catfish contributes to the findings of differential expression profiles in different tissues (Milev-Milovanovic *et al*., 2006).

IFN-γ could increase the expression of genes that related to the activity of macrophages in the turbot if infected with bacteria (Pereiro *et al*., 2016). The recombinant goldfish IFN-γ (rgIFN-γ) was primed with immune cells to increase the phagocytic and nitric oxide responses of macrophages and enhanced respiratory burst

responses. In addition, expression levels of several proinflammatory genes were increased in treatment of goldfish macrophages with rgIFN-γ induction (Grayfer and Belosevic, 2009). The intramuscular injection of pMCV1.4-IFN-γ could affect the transcription of macrophage-colony stimulating factor (csf1), which was directly related to the activity of IFN-γ. Previous studies showed that the expression levels of pro-inflammatory cytokines and type I IFN genes were higher in the turbot, infected with pMCV1.4-IFN-γ and Viral Hemorrhagic Septicemia Virus (VHSV) than those in fish infected without the expression plasmid (Pereiro *et al*., 2016).

Recombinant IFN-γ (rIFN-γ) plays an important role in the immune response. It has been used to enhance the resistance to pathogens in some animals and fish (Zou *et al*., 2005; Robertsen, 2006; Grayfer and Belosevic, 2009; Swain *et al*., 2015). But there was a little with respect to the report of the biological activity of the rIFN-γ of *Takifugu rubripes* and its application effect need further exploration.

T. rubripes is one of marine commercial fishes in Asia. However, various infectious diseases, which were caused by viruses, bacteria and parasites, have hindered its production and caused economic losses (Du, 2003; Cui *et al*., 2014; Qin *et al*., 2016) and can lead to major economic loss. To solve the problems underlying infectious diseases in *T. rubripes*, it is of interest to make an efficient rIFN-γ protein. In this study, IFN-γ gene was cloned from *T. rubripes* and expressed in *Pichia pastoris*. The molecular weight, concentration and bioactivity of rIFN-γ were measured. We also identified the rIFN-γ protein of *T. rubripes* could stimulate WISH cell proliferation, which was associated with recombinant protein concentration. These results provide a basis for application of the rIFN-γ protein as a vaccine candidate against pathogen infection in aquaculture.

Materials and Methods

Construction of Recombinant Expression Plasmids

IFN-γ gene of *T. rubripes* was queried from NCBI (Gen Bank: AJ616216.2). Based on the codon bias of the methylotrophic yeast *P. pastoris*, the IFN-γ mature peptide was designed. The plasmid harboring the IFN-γ gene was transformed into *TOP* 10 cells, and then incubated in LB agar with 100 μg/mL Zeocin. We selected the positive recombinant cells and cultured them in LB medium with 100 μg/mL Zeocin. The positive recombinant vector was extracted and named pPICZαA-IFNγ. The plasmid pPICZαA-IFN-γ was linearized by *Sac* I quick cut enzyme and transformed into *P. pastoris* competent cells by electroporation. The transformants were selected on the plate containing Zeocin (100 μg/mL). To select putative multicopy recombinants, the transformation max was plated on a high concentration of Zeocin (1000–2000 μg/mL). Eight strains were randomly selected and inoculated into YPDS liquid medium until OD_{600nm} = 1.0 to 2.0. The pPICZαA-IFN-γ-F, and PICZαA-IFN-γ-R were used as primers for PCR amplification to confirm that the target gene sequence was successfully integrated into the GS115 genome. The primers (FP: 5' GCTGCTAAAGAAGAAGGGGT 3' and RP 5' AGATCCTCTTCTGAGATGAG 3') were used for confirmation. PCR products were sent to Takara biotechnology (Dalian) company for sequencing.

Expression and Identification of Recombinant Protein

P. pastoris cells, harboring recombinant plasmids pPICZαA-IFN-γ and the negative control of pPICZαA, were incubated with shaking at 250 rpm in BMGY containing 100 μg/mL Zeocin at 37°C until the OD_{600nm} = 2 to 6. The cells were centrifuged at 12,000 rpm for 3 min at 4℃. Then, the cells were washed in sterile water and diluted several times in fresh BMMY (Buffered Methanol-complex Medium) until the OD_{600nm} = 1. The expression of IFN-γ was induced with 1% methanol final concentration and cultured in a baffled flask and kept in an incubator for 96 h at 28°C. The supernatant of the culture was harvested by centrifugation at 12,000 rpm for 3 min at 4°C. These samples were dialyzed in PBS and condensed in a 3K hyperfiltration tube. In addition, the protein was dissolved by 75% ammonium sulfate and dialyzed at 48 h in PBS. The concentrates of rIFN-γ were analyzed by SDS-PAGE gel electrophoresis. The final concentration of rIFN-γ was quantified by the Bradford method.

Mass Spectrometry (MS) Analysis

To identify the rIFN-γ recombinant protein, we used the method of digestion and MS analysis. The MS analysis was performed using Eksigent nanoLC-Ultra™ 2D, TripleTOF 5600 and Mascot 2 (Katayama *et al*., 2001; Ens and Standing, 2005).

Cell Culture and Bioactivity Assessment

Adherent WISH cells were grown in the culture medium and cultured at 2 to 3 times a week with the dilution ratio of 1:2–1:4. The adherent cells were inoculated in a 96-well cell culture plate at a concentration of 2.5×10^5 – 3.5×10^5 cells per well. The suspension of WISH cells was cultured for 4–6 h at 37℃, 5% CO_2 until the cells grew in a monolayer. The standard IFN and rIFN-γ were diluted 4^1, 4^2, 4^3, 4^4, 4^5, 4^6, 4^7 and 4^8 times. The supernatant of negative strains was treated similarly as the negative control. Each sample was performed in triplicate. The cells treated with the standard and rIFN-γ protein were incubated for 24 h. Then, the cells were infected with 100 μL/well of 100 $TCID_{50}$ units with Vesicular stomatitis virus (VSV) for 24 h, and the inoculum was removed. Each sample contained 20 μL of MTT and was cultured for 4 h. Then, 150 μL of DMSO was added

to the sample with shaking for 10 min after culturing for 2 h. The absorbance of the cells was measured at the wavelength of 570 nm. The biological activity of IFN-γ protein was then calculated.

Statistical Analysis

All data were conducted by SPSS with means ± SDs from three independent experiments. Statistical significance was evaluated with $P < 0.05$.

Results

Construction and Expression of Recombinant Protein IFN-γ

The *IFN-γ* sequence was 570 bp and contained a signal peptide of 66 bp and a mature peptide of 504 bp. This peptide was encoded from 189 amino acids, which contained a signal peptide of 22 amino acids and mature peptide of 167 amino acids. The molecular mass of *T. rubripes IFN-γ* was included as ssDNA (154.89) and dsDNA (309.49). According to the selectivity of the expression vector, the codon of the mature peptide of the *IFN-γ* gene of *T. rubripes* was optimized. We found that the similarity between the optimized nucleotide sequence and the *IFN-γ* gene sequence was 70%. Meanwhile, the amino acid sequence was unchanged. An Xho I site and kex2 sequence were added at the N-terminus of the optimized target gene sequence. In addition, a terminal codon TAA and the *Xba* I restriction site were followed by the sequence encoding IFN-γ protein in the sense strand. The sequence was synthesized and connected to the MCS region of the plasmid pPICZαA. The recombinant vector was digested by double restriction enzymes to obtain the fragments of 3.5 kbp and 500 bp (Fig. 1). The *IFN-γ* gene was successfully cloned in the pPICZαA expression vector. pPICZαA, an excretion vector, could directly secrete the protein product of into the culture medium, which could reduce the loss of purified protein. In the processes of optimizing the gene sequence and constructing the expression vector, we only added the protein-cutting enzyme site of the kex2 protein upstream of the *IFN-γ* gene, but downstream of the gene, the protein-cutting enzyme site was not added because of the effect of the protein secondary structure and spatial structure. The pPICZαA vector contained an α-factor signal sequence that has the preliminary cleavage of the signal sequence by the kex2 gene product. The final kex2 cleavage occurred between the arginine and glutamine in the sequence of Glu-Lys-Arg * Glu-Ala-Glu-Ala, where * is the site of cleavage. Our results support the notion that upstream of the IFN-γ gene with the sign of the enzyme site was able to completely express, but the process of the separation and purification were difficult.

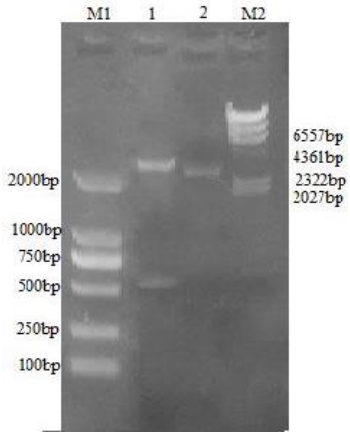

Fig. 1: Agarose gel electrophoresis after double digestion. M1, DNA 2000 marker; M2, λ-HindⅢ marker; Lane 1, pPICZαA-IFNγ with double enzyme digestion; Lane 2, pPICZαA-IFNγ without double enzyme digestion

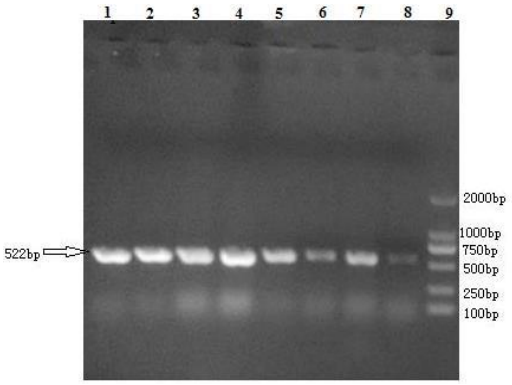

Fig. 2: The resulting PCR products. Lanes 1 to 8, the fragments of PCR amplification; Lane 9, DNA 2000 Marker

Identification and MS Analysis of rIFN-γ

The recombinant plasmid was linearized with *Sac* I quick cut enzyme and transformed into the *P. pastoris* competent cells that were cultured in YPDS medium with 100 μg/mL Zeocin. Moreover, the strains had grown in the plate. The genomic DNA of *P. pastoris* was extracted and was used for template PCR amplification and sequencing. The PCR products were 504bp, which was consistent with the size and nucleotide sequence of the inserted DNA (Fig. 2 and 3). The strains were cultured on BMMY for expression with methanol as the inducer. To obtain a high level of expression of the recombinant IFN-γ, it was then induced in 1% methanol with shaking at 96 h at a speed of 200 rpm at 28℃.

A 24 kDa expressed protein was observed *via* 12% SDS-PAGE (Fig. 4). The final concentration of recombinant protein was 0.242 mg/mL. This result potentially met the requirements of the next experiment. Based on MS analysis, the peptide score was distributed in 0~15 and the score of

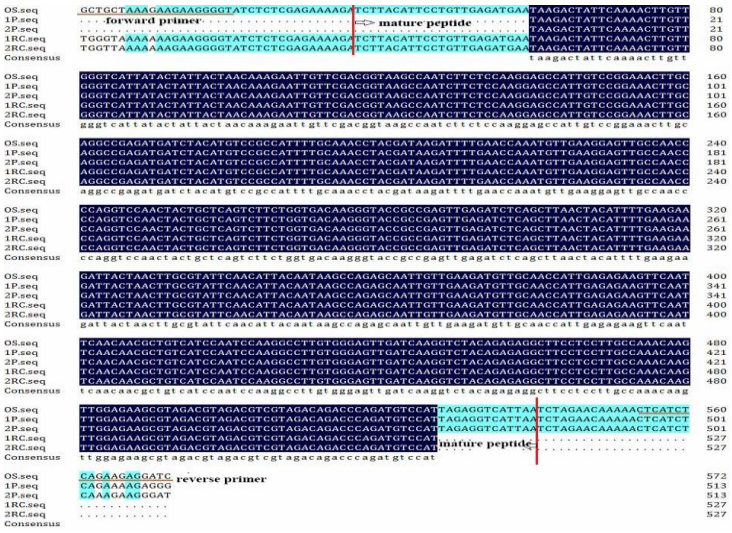

Fig. 3: Results of Eukaryotic genome sequencing of PCR and comparison with the original sequence. OS is original sequence; 1 P and 2 P are the sequences of the sample by forward sequencing; 1 RC and 2 RC are the sequences of the sample by reverse complementary sequencing

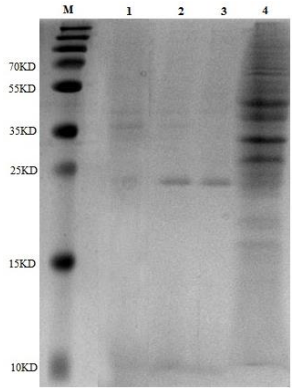

Fig. 4: The expression of Taki-IFNγ recombinant protein in different concentrations of methanol. M, protein ladder marker; Lane 1, supernatant with a methanol concentration of 1.5%; Lane 2, supernatant with a methanol concentration of 1.0%; Lane 3, supernatant with a methanol concentration of 0.5%; Lane 4, supernatant with a methanol concentration of 1.0% after crushing

the target protein was 15. The protein fragment was compared with the known fragment from BLAST, the coverage rate was 28%, and the red region is the peptide segment of the matching. The results of MS analysis for rIFN-γ are shown in Fig. 5. A total of six peptides were matched, including the 87– 94, 101–118, 113–118, 119–130 and 157–165 (Table 1). Thus, the recombinant protein was confirmed to be rIFNγ in this experiment.

Verification of the Biological Activity of rIFN-γ

To determine the bioactivity of rIFN-γ, WISH cells were incubated overnight with rIFN-γ culture supernatants. These cells were subsequently challenged with VSV and observed 24 h later for the cytopathic effect. According to the OD_{570nm} value of the protein sample, we made cell proliferation curves and analyzed the curves. We concluded that rIFN-γ protein had the same tendency with standard samples with regard to cell proliferation. The percentage of cell proliferation was decreased with an increasing dilution rate of the protein. This phenomenon indicated that rIFN-γ protein had an anti-virus function and could promote the proliferation of cells. Eight adjacent points were selected from the OD_{570nm} value of the protein sample to make a regression line. The results of the WISH cell proliferation percentage are shown in Table 2. The bioactivity of rIFN-γ protein in *P. pastoris* was 2018 IU/mL, which was higher than other recombinant IFN proteins (Zhang *et al*., 2012). The bioactivity of IFN-γ was confirmed against vesicular stomatitis virus *in vitro*, and the inhibitory concentration of rIFN-γ was found to be as low as 100 ng/mL (Mallick *et al*., 2011). The activity of rIFN-γ was confirmed by its ability to up-regulate class II major histocompatibility complex (MHC) on cells of the porcine monocytic cell line 3D4/31 (Rupa *et al*., 2008).

Discussion

In this study, IFN-γ gene was cloned from *T. rubripes* and expressed in *P. pastoris*. The molecular weight, concentration and bioactivity of rIFN-γ were measured. We also identified this rIFN-γ could stimulate WISH cell proliferation, which was associated with recombinant protein concentration. These results provide a basis for

Table 1: The distribution of peptide by MS analysis

Start - End	Observed	Mr (expt)	Mr (calc)	ppm	Miss Sequence
87 - 94	490.2797	978.5449	978.5386	6	R.SQLNYILK.K
101-118	378.5415	2265.2054	2265.2303	-11	R.IQHYNKPEQLLKMLQPLR.E
113-118	387.2190	772.4234	772.4265	-4	K.MLQPLR.E
113-118	387.7107	773.4068	773.4105	-5	K.MLQPLR.E
119-130	688.8588	1375.7030	1375.7096	-5	R.EVQFNNAVIQSK.A
157-165	589.3167	1176.6189	1176.6033	13	R.RRQTQMSIR.G

Table 2: The OD_{570nm} values of the rIFN-γ and the standard IFN-γ Sample and the WISH cell proliferation percentage

log4 dilution ratio	OD_{570nm} * means ± SD	Cell proliferation percentage (%)	OD_{570nm} ** means ± SD	Cell proliferation percentage (%)
1	0.96 ± 0.01c	73.4%	1.03 ± 0.11ab	85.1%
2	1.31 ± 0.06a	100.0%	1.21 ± 0.13a	100.0%
3	1.13 ± 0.09b	86.0%	1.19 ± 0.16a	98.6%
4	0.94 ± 0.05c	71.8%	0.98 ± 0.06b	81.4%
5	0.93 ± 0.01c	70.6%	0.89 ± 0.06b	73.6%
6	0.86 ± 0.03c	65.6%	0.81 ± 0.02c	67.2%
7	0.69 ± 0.01d	52.4%	0.79 ± 0.01c	65.4%
8	0.68 ± 0.01d	51.5%	0.76 ± 0.01c	62.7%

Note: *: rIFN-γ; ** standard IFN-γ Sample
abcd Means within the same column of each locus with different superscripts are significantly different ($P < 0.05$)

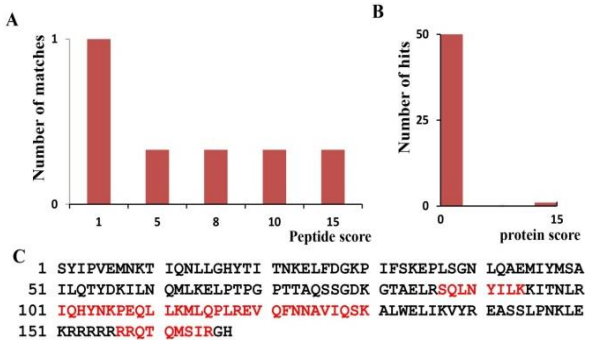

Fig. 5: MS analysis for rIFNγ recombinant protein. A: Peptide score distribution. Ions score is−10 log (P), where P is the probability that the observed match is a random event. Scores > 0 indicate identity or extensive homology ($p < 0.05$). B: Score distribution for family members in the first 50 proteins. Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein families. C: Matched peptides shown in bold red

application of the rIFN-γ protein as a vaccine candidate against pathogen infection in aquaculture.

The rIFN-γ may be potentially the most effective immune inducer among the tested cytokines. Therefore, rIFN-γ could be an efficient technique for disease prevention in fish. Presently, the recombinant interferon protein has been applied in *in vivo* and *in vitro* tests. The expression levels of the *CXCR3a* and *CXCR3b* genes increased in the immune-related cells stimulated with resulting rIFN-γ *in vitro* (Coelho *et al*., 2008; Xu *et al*., 2014). In infected macrophages, IFN-γ can increase the accumulation of the immune proteins. Guanylate Binding Proteins (GBPs) were induced by and type I IFN can inhibit it (Wallet *et al*., 2017). In addition, rIFN-γ significantly induced the expression of interleukin (IL) genes, which can lead to inhibition of Th17 differentiation. There was substantial evidence that rIFN-γ in pathogenic Th17 cells played important roles in chronic inflammatory processes and in the induction of autoimmune diseases (Khayrullina *et al*., 2008). Additionally, IFN-γ-mediated host protection against *F. novicida* is largely GBP-dependent and inflammasome-independent *in vivo* (Wallet *et al*., 2017). Furthermore, the survival rate in the rIFN-γ-injected group was significantly higher compared with the group treated with *E. tarda* (Jung *et al*., 2012). Puffer fish that received rIFN-γ injection had an elevated phagocytic activity and lysozyme. Additionally, IFN-γ also regulated the expression of miRNA. For example, the expression levels of miR-145 and miR-29 were changed after rIFN-γ1 and rIFN-γ2 treatment in *Tetraodon nigroviridis.* These two miRNAs can target immune genes including *cytokine signaling (SOCS)7*, *c-Myc*, and *Stat1*. These data suggest that IFN-γ also affects the *T. nigroviridis* immune system by the regulation of miRNA expression (Yi *et al*., 2014). In addition, IFN-γ may potentially be the most effective immune inducer among the tested cytokines (Biswas *et al*., 2016). In this study, the rIFN-γ of *T. rubripes* could enhance the resistance of cell to VSV. These results could provide a base for further study on the diagnosis and evaluation if it could be an efficient technique for disease prevention in fish.

Conclusion

In this study, IFN-γ was cloned from *T. rubripes* and expressed in *P. pastoris*. We obtained the molecular weight of rIFN-γ was 24 kDa. The bioactivity of rIFN-γ was 2018 U/mL and the concentration of the rIFN-γ protein was 0.242 mg/mL.

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