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Characterization of the biological activities and physicochemical characteristics of recombinant bovine interferon-α_{14}

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ABSTRACT

A BoIFN-α gene that included signal sequence was amplified from bovine liver genomic DNA. The gene was named BoIFN-α_{14} according to the position at which the encoded gene of the bovine IFN was located in the bovine genome. The sequence included a 23-amino acid signal peptide and 166-amino acid mature peptide. The structural characteristics and phylogenetic relationships of the BoIFN-α_{14} gene were analyzed. A recombinant mature BoIFN-α_{14} (rBoIFN-α_{14}) was expressed in the yeast *Pichia pastoris*. Antiviral activity, antiproliferation activity and physicochemical characteristics were determined *in vitro*. Recombinant BoIFN-α_{14} exhibited a considerable antiviral activity against Vesicular stomatitis virus (VSV), which was neutralized by a rabbit polyclonal anti-rBoIFN-α antibody, and could inhibit cell proliferation. It was also found to be highly sensitive to trypsin and stable at pH 2.0 or 65 °C. This study revealed that rBoIFN-α_{14} has the typical characteristics of IFN-α and can be used for both research and industrial application.

1. Introduction

Interferons (IFNs) form an important group of cytokines that are now recognized as key components of the innate immune response and the first line of defense against virus infection (Sadler and Williams, 2008). It was first reported by Isaacs and Lindenmann that chick cells infected by influenza virus produced IFNs that mediate the transfer of a virus-resistant active state against both homologous and heterologous viruses in 1957. Now, IFNs are generally classified into type I, II and III (Sergei et al., 2003), and IFN-α belongs to type I IFNs. IFN-α is encoded by a family of closely related intronless genes in all mammalian species (Weissmann and Weker, 1986). Multiple genes that encode IFN-α subtype have been identified in several mammalian species. The Bovine IFN-α (BoIFN-α) is encoded by a multigene family with approximately 10 to 12 distinct BoIFN-α proteins (Capon et al., 1985; Velan et al., 1985). Overall the BoIFN-α subtypes show more than 92% nucleotide sequence identity and at least 90% amino sequence identity. BoIFN-α contains 162 to 168aa, whereas BoIFN-α contains approximately 65% homology as compared to human IFN-α, and approximately 57% homology as compared to mouse IFN-α (Oritani et al., 2000).

Despite the high degree of identity among the IFN-α subtypes from the same species, differences in function and magnitude of biologic activities are often observed. Different subtypes of HuIFN-α exhibit various antiviral activities. Among all the subtypes, HuIFN-α8 is reportedly the most potent, whereas HuIFN-α1 provides the lowest antiviral activity (Fostern et al., 1996). Several authors have reported that MuIFN-α4 was 5 to 10 times more active than MuIFN-α1 (Van Heuvel et al., 1998). Thus, the biological activities among these proteins vary even though they are from the same species, and each member of the IFN-α family should be characterized.

In this study, a Bovine IFN-α gene, with a sequence 100% identical with that of NCBI ID XM_005199122.1 IFN-αD [*Bos taurus*], was amplified. The IFN-αD gene was first obtained in 1985, but information on its characteristics remains unavailable, and detailed information on other BoIFN-α genes is also unavailable (Velan et al., 1985). The nomenclature of BoIFN-α_{14} was based on its position in the bovine genome (Zhao et al., 2009). Sequence secondary structural analysis and phylogenetic relationships of the BoIFN-α_{14} gene were conducted. The gene that encodes the mature peptide of BoIFN-α_{14} was cloned and expressed by *Pichia*

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pastoris/pPICZaA. The biological activities and physicochemical characteristics of purified rBoIFN-α_{14} were determined *in vitro*. The results clearly showed that rBoIFN-α_{14} exhibits significant antiviral activity against VSV in MDBK cells, comparable antiproliferation activity in MDBK cells in a dose-dependent manner, and that it possesses the typical characteristics of IFN-α. Thus, BoIFN-α_{14} is a potential antiviral agent against infectious bovine diseases.

2. Materials and methods

2.1. E. coli and yeast strains

Escherichia coli DH5α (New England Biolabs, UK) was used to construct the recombinant plasmid. The yeast *P. pastoris* GS115 (Invitrogen, USA) was used as a host strain to express the recombinant plasmid.

2.2. Cells and viruses

Madin–Darby bovine kidney (MDBK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL). The vesicular stomatitis virus (VSV, Indiana strain) was propagated in MDBK cells and stored at −70 °C. The virus was purchased from China Institute of Veterinary Drug Control. The 50% tissue culture infectious dose ($TCID_{50}$) of the virus in MDBK cells was determined by serial dilution.

2.3. Rabbit polyclonal anti-BoIFN-α antibody

The rabbit polyclonal anti-BoIFN-α antibody was prepared in our laboratory using recombinant BoIFN-αA as antigen.

2.4. Cloning of BoIFN-α_{14} gene and sequence characteristics analysis

The BoIFN-α_{14} gene, which contained signal sequences, was amplified from bovine liver genomic DNA by PCR. The PCR program was 94 °C for 5 min, 30 cycles of 94 °C for 50 s, 63 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min. The PCR product was inserted into pMD18-T vector (TaKaRa, Kyoto, Japan) and the nucleotide sequence was determined. Its secondary structure elements were analyzed using the algorithms available from NPS (http://www.npsa-pbil.ibcp.fr). The putative glycosylation sites of BoIFN-α_{14} gene were predicted using the NetNGlyc website (http://www.cbs.dtu.dk/services/NetNGlyc) and YinOYang (http://www.cbs.dtu.dk/services/YinOYang/).

2.5. Phylogeny reconstruction

The multiple sequence alignment of BoIFN-α was compared with that of their counterparts using the ClustalX program. The sequences used in the comparison were retrieved from GenBank. A phylogenetic tree was constructed using MEGA5.0 and the neighbor-joining method with a bootstrap of 1000 repetitions (Tamura et al., 2011). The IFN-β of horse and sheep was chosen as outgroups.

2.6. Subcloning of BoIFN-α_{14} mature peptide gene and construction of secreted expression vector

A pair of primers was designed to amplify the gene that encodes and expresses mature rBoIFN-α_{14} peptide in yeast *P. pastoris*. The primer sequences are shown in Table 1. The underlined sequence in the forward primer indicated the *Xho* I restriction site, which was in-framed with the start codon of α-factor secretion signal on pPICZαA. The reverse primer indicated the *Eco*R I restriction site, which was denoted by the underlined portion. The PCR program used to amplify the BoIFN-α_{14} gene was as follows: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min. The final PCR product was digested with *Xho* I and *Eco*R I and cloned into the *E. coli*/*P. pastoris* shuttle vector pPICZαA (Invitrogen, USA), which was predigested with similar enzymes. The construct was designated as pPICZαA–BoIFN-α_{14}.

Table 1
Sequences of the primers.

Primers	Sequences
Forward	5′ AGCTCTCGAGAAAAGATGCCACCTGCCTCACTCC3′
Reverse	5′ CCGGAATTCAACCAGGTGTGTGTCAGTCC3′
5′AOXI	5′GACTGGTTCCAATTGACAAGC3′
3′AOXI	5′GCAAATGGCATTCTGACATCC3′

The sequences underlined with single line, double lines denoted the cleavage sites of *Xho* I and *Eco*R I, respectively; the shadowed sequences denoted the cleavage sites of Kex2.

2.7. Transformation and screening of Pichia pastoris

The constructed recombinant plasmid pPICZαA–BoIFN-α_{14} was linearized by *Pme* I. The linearized plasmid (10 μg) was electroporated in 100 μL competent *P. pastoris* GS115 cells according to the user's manual provided by Invitrogen with minor modifications (Sambrook et al., 1992). The transformants were grown and selected at 30 °C on the YPDS agar plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, 20 g/L agar and 1 M sorbitol) with 100 μg/mL Zeocin™ for 3 to 4 days. The boiling-freezing-boiling method was used to prepare recombinant *P. pastoris* genomic DNA, which was used as template in PCR identification (Ju et al., 2003). The PCR primer set included the universal primers 5′AOX1/3′AOX1 as well as the specific forward and reverse primers. The parameters on the PCR program were set for PCR amplification. The detected positive transformants were used in subsequent expression experiments. The sequences of the above primers are shown in Table 1.

2.8. Expression and purification of rBoIFN-α_{14} in P. pastoris

The expression of pPICZαA–BoIFN-α_{14} in *P. pastoris* was conducted in a flask as described (Hou et al., 2011; Huang et al., 2012). The entire culture supernatant was harvested after a 96 h incubation. The protein was obtained from the supernatant, purified by ammonium sulfate precipitation, and dialyzed for 2 days against PBS solution. The purified rBoIFN-α_{14} protein was concentrated, filtered through a 0.22 μm filter and stored at −70 °C for further use. The final protein concentration was determined using a BCA Protein Assay Kit (Pierce, USA).

2.9. SDS-PAGE and Western blot analysis of rBoIFN-α_{14}

SDS-PAGE analysis was conducted using 5% (v/v) stacking gel, 15% (v/v) resolving gel, and stained by Coomassie Brilliant blue G-250 (Cao, 2004). For Western blot analysis, after SDS-PAGE, proteins were transferred to the nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBST (PBS, 0.5% Tween-20, pH 7.4) at 37 °C for 2 h, incubated with 1:250 (v/v) rabbit polyclonal anti-bovine IFN-α antibody diluted in PBST at 37 °C for 2 h, washed three times using PBST at 10 min interval, and then incubated with 1:5000 (v/v) goat anti-rabbit IgG conjugated to horseradish peroxidase at 37 °C for 1 h. Finally, the membrane was visualized with 4-chloro-1-naphthol (4-CN).