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Characterization of Interferon-α1 of Yak

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Abstract: The yak (*Bos grunniens*) is an important grazing livestock in Western of China. In this study, the full length of *yIFN-a1* gene was cloned then the recombinant yIFN-a1 was expressed in yeast expression system. *In vitro* bioactivity of IFN-α1 was determined by inhabition infection BHK cell against Vesicular Stomatitis Virus (VSV). The results showed that cDNA contains an Open Reading Frame (ORF) of 570 base pairs encoding a protein of 189 amino acid (aa) precursor with a signal peptide of 23 aa. The homology of the yIFN-a1 was up to 65.3~94.4% with other species at the amino acid levels. The recombinant yIFN-a1 was recognized in Western blot by mAb against human IFN-α1. The activity unit of the purified recombinant yIFN- a1 protein was 1.16×10^5 IU mg^{-1} by against Vesicular Stomatitis Virus (VSV) antiviral assay *in vitro*. This made a basis for the development of anti-virus medicine of yak in the future.

Key words: Yak (*Bos grunniens*), interferon-α1, cloning, antiviral activity assay, *Pichia pastoris*

INTRODUCTION

The yak (*Bos grunniens*) is an important grazing livestock in Western of China. Yaks are chiefly populated in the cold, high-altitude areas (about 3000-4000 m above the sea level) in Qinghai, Tibetan, Sichuan, Gansu, Xingjiang and Yunnan provinces. However, there are more 20 kinds of infectious diseases in yak, it is a threat to promote healthily development of yak culture industry. Therefore, it is necessary to study the IFN for application in yak defence disease.

Interferons (IFNs) are not only powerful antiviral agents but also act as cytokines, possessing activities for controlling cellular functions related to growth, differentiation and immunoregulation. IFNs are divided type-I and type-II, type I IFNs include multiple Interferon alpha (IFN-α) subtypes, a single IFN-β, IFN-ω and IFN-t while type II IFNs include only one member, IFN-γ (Wonderling *et al*., 2002). With the development of engineering technology, *IFN-α* gene has been cloned from a number of species including human, porcine (Chinsangaram *et al*., 2003; Lefevre and Bonnardiere, 1986) horse, rat and so on. Cattle IFN-α is encoded by a multigene family with approximately 10-12 members and cloned from cow cDNA library using human *IFN-α* gene as a probe which appear to four distinct families (A, B, C, D) on the basis of sequence indentity. IFN-E of cattle has been cloned from the epithelium of a rotavirus infected calf and expressed in *E. coil* (Chaplin *et al*., 1996). The interferon G, F, H subtype has been cloned from the gut epithelium of rotavirus infected calves (Paul *et al*., 1996). At present, more 8 subtype of IFN-α have been cloned and it's biological function were researched. Some research showed that recombinant cattle IFN-α could inhibit the replication of FMDV, VSV, BVDV, TEGEV and PRRSV and so on.

IFN a1 is one of genes of IFN-α subtype family is also known as Interferon D (IFND) has been used wide research for clinical interest. Recent reports have demonstrated the efficacy of recombinant IFN-α1 in the treatment of viral diseases in animals as well as in humans. In humans, IFN-α1 has extensively been used for treatment of patients with some types of cancer and viral diseases (Ferrantini *et al*., 2007). In cats, IFN-α1 subtypes demonstrated significant anti-viral activity (Wonderling *et al*., 2002). In bovine, research of IFN-α1 mainly trend to the yellow cattle and cow but yak IFN-α1 have no report.

In this study, the yak Interferon (*yIFN-α1*) gene was cloned and expressed in *P. pastoris* expression system. The anti-Vesicular Stomatitis Virus (VSV) antivity was researched *in vitro*. Additionally, comparison between *IFN-α1* genes of yak and other mammalian species was conducted. This study made a basis for providing a gene engineering interferon to the yak.

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MATERIALS AND METHODS

Reagents bacteria: Catrimox-14™ RNA extraction kit, AMV, Taq DNA polymerase, dNTP, EcoR I, Not I and RNAase inhibitor (Dalan TaKaRa Corporation, China), pGEM-T easy vector and pPIC9k vector (Promega Corporation, USA), gel DNA purification kit and lymphocyte isolation reagent (Boda Corporation in Beijing, China), PHA and LPS purchased from Sigma Corporation (USA) were used. Bacteria seed (JM109) stored in the lab.

Animals samples: Blood samples were collected from yaks in Gansu province, China.

RNA isolation and cDNA synthesis: Peripheral Blood Monouclear Cells (PBMC) were separated from heparin-containing yak blood with lymphocyte isolation reagent and its concentration was adjusted to 1×10^{7} mL^{-1}, followed by combined stimulation with ConA. The PBMC were washed with PBS by centrifugation at 2000 rpm for 7 min. Total RNA was extracted from the collected cells with Catrimox-14™ RNA kit (Dalan TaKaRa Corporation, China). cDNA was synthesized at 42°C using oligo (dT)-adaptor primer and Avian Myeloblastosis Virus (AMV) reverse transcriptase.

Cloning of *yIFN-a1* gene: To amplify the entire coding region of yak IFN-α1, the sequence primersforward: 5'-ATG GCC CCA GCC TGG TCC TT-3' and reverse: 5'TCA GTC CTT TCT CCT GAA ACT CTC C-3') designed were based on the published mammals sequences: *Bos taurus* IFN-a1 (A10045), *Ovis aries* interferon-α1 (AY802984), *S. scrofa domestica* IFN-α1 (AY345969) with the oligo6.0 Software (National Biosciences, Plymouth, MN, USA). An aliquot (0.5 μL) of synthesized cDNA was used as a template to clone yak IFN-α1 cDNA using the following Polymerase Chain Reaction (PCR). The amplification profile included one initial denaturation step at 95°C for 5 min then 35 cycles of 94°C for 40 sec, 54°C for 50 sec and 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 1% agarose gel, cloned into the T-Easy vector (Invitrogen, America) and subsequently transformed into JM109 *E. coli* cells. Recombinant plasmids were selected by blue white selection and sequenced (TakaRa Biotechnology).

Phylogenetic analysis: The full-length multiple alignment of the IFN-α1 sequences from yak was compared with other earlier known IFN-α1. Nucleotide sequences and amino acid sequences from other species retrieved from the NCBI GenBank were analyzed using the MEGA 4.0 Software Version 4.0 Software (Tamura *et al*., 2007). A Neighbor Joining (NJ) phylogenetic tree was constructed using MEGA Software Version 4.0 (Tamura *et al*., 2007). The reliability of the branching was tested using bootstrap re-sampling (1000 pseudo-replicates).

Construction of expression vector pPIC9K-yIFN-a1: According to the codon bias of *P. pastoris*, the expression primers were designed based on the sequence of *yIFN-a1* gene. The forward primer, 5'CGA GAATTC ATG TGC CAC GTG CCA CAC AC3' (the underlined sequence is the EcoR I site) and the reverse primer, 5'AAT GCGGCCGC TCA GTC CTT TCT CCT GAA ACT CT-3' (the underlined sequence is the Not I site). The fragment gene with the length of 504 bp was subcloned into expression vector pPIC9K (Invitrogen, USA). The mutation-free recombination plasmids were confirmed by sequencing.

Transformation of *P. pastoris*: After linearization with Sal I, the pPIC9k-yIFN-a1 recombinant plamid was purified and transformed into *P. pastoris* strain GS115 according to the manual (Invitrogen, USA). The transformant cells were plated on a MD medium plate (100 mM potassium phosphate, pH 6.0/1.34% yeast nitrogen base/4×10^{5}% biotin/1% glycerol/1.5% agar). After incubation at 28°C for 3 days to analyze the copy number of the clones, about 100 colonies were replated on a YPD medium (1% yeast extract/2% peptone/2% glucose) containing 0.5, 1 and 2, 4 mg mL^{-1} of Zeocin (Amresco, USA). Some colonies appeared on the YPD plate containing 4 mg mL^{-1} of zeocin which was performed using PCR analysis with yIFN-a1 specific primer (5' CGA GAATTC ATGTGCCACGTGCCTCACAC3') and 3'AOX1 primer (5'GCAAATGGCATTCTGACATCC3') for identification of positive clones.

Expression and purification of yIFN-a1: The positive colonies were grown overnight in 15 mL BMGY medium (1% yeast extract/2% peptone/100 mM potassium phosphate, pH 6.0/ 1.34% yeast nitrogen base/4×10^{5}% biotin/1% glycerol) at 28°C. After centrifugation, the harvested cells were grown in 25 mL of BMMY medium (1% yeast extract/2% peptone/100 mM potassium phosphate, pH 6.0/1.34% yeast nitrogen base/4×10^{5}% biotin/1% methanol). The cultures were fed with 2.5 mL of 10% methanol at 24 and 48 h postinduction. After 3 days of culture, the entire culture supernatant was harvested and analyzed by Sodium Dodecyl Sulfate Polyacrylamide

Gel Electrophoresis (SDS-PAGE). Western blotting was performed with a mouse anti-human IFN-α1 mouse monoclonal antibody.

The positive clones that could expressed the yIFN-a1 protein in high level was induction in a large scale. The supernatant was purified by Sephadex G-100 gel chromatography, eluted with 0.01 M phosphate-buffered saline (PBS; pH 7.2) at 0.5 mL per min. The purity and concentration of the purified protein were determined using SDS-PAGE and quantitated protein BCA kit (Beyotime institute of biotechnology, Beijing).

Antiviral assay: The antiviral activity of the yak IFN-α1 protein was determined by crystal violet assay which detected the reduced Cytopathic Effect (CPE) of Vesicular Stomatitis Virus (VSV) in BHK cells (Wonderling *et al*., 2002). Typically, 2×10^4 BHK cells were added to 96 well plates per well in 100 μL Dulbecco's Modified Eagle Medium (DMEM) (Heclony, American) with 5% Fetal Calf Serum (FCS) and incubated at 37°C with 5% CO_2 in a humidified incubator until the cells were 80-85% confluent 10 fold serially diluted purified protein was added into 96 well plates in triplicate. Untreated cell control and virus control experiments were performed in parallels. Human IFN-α1 (2.5×10^7 units/mg ebioscience, US) was used as standard control. After incubation for 24h at 37°Cand 5% CO_2, the supernatants of the cultures were removed and the cells were washed twice with PBS and inoculated with 100 μL of 100 $TCID_{50}$ VSV. The plates were gently washed three times with PBS after the cells incubated for 20 h.

Cells were fixed in 5% (v/v) formalin and stained with 0.1% (w/v) crystal violet. The cells were then washed with de-ionized water and allowed to dry. The crystal violet was dissolved with 100 μL of methanol and the absorbance values were measured at 570 nm with a microtiter reader.

RESULTS

Sequence analysis of yak *IFN-α1* gene: The yak *IFN-α1* gene was amplified by RT-PCR from cDNA derived from RNA that was isolated from yak PBMC. Sequence analysis showed that the obtained *yIFN-a1* gene contains an open reading frame of 570 (bp) long with encoding a 189 amino-acid precursor. Based on the amino acid sequence, the SignalP 3.0 server predicted the location of the signal peptide cleavage between amino acids 22 and 23 indicating a mature protein of 166 aa in length with a predicted molecular weight of 20 kDa. In comparison of cloned yak IFN-α1 with cow, sheep, porcine *IFN-α1* genes, the identity of amino acid were 90.5, 88 and 65.3%, respectively and the nucleotide acid identity were 94.4, 94.7 and 80.7% (Fig. 1).

Phylogenetic analysis: The neighbor-joined trees (Fig. 2) revealed that yak IFN-α1 sequences were classified into two groups of mammalians and birds, respectively. The mammalians group were furthermore divided into two subgroups, among them, yIFN-a1 is most closely related to a IFN-α1 from the cow and sheep.

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yak          MAPAWSFLLA LLLLSCNAIC SLGCHVPHTH SPPNRRVLTL LRQLRRVSPS SCLQDRNDFA
Bos_taurus   .........S .......... .....L.... .LA.....M. .Q........ .........E
Ovis_aries   ---------- ---------- ---..L.... .LA....... .Q...A.... ......K...
S.scrofa_d   ...TSA..T. .V........ ....DL.Q.. .LAHT.A.R. .A.M..I..F ...DH.R..G

yak          FPQKALGGSQ LQKAQAISVL HEVTQHTFQL FSTEGSATMW DESLLDKLHA ALDQQLTDLQ
Bos_taurus   .L.E...... .......... .......... ......PAT. .K.....R.. ..........
Ovis_aries   .......... ...V.T.... .......... .......AA. .Q.....R.. ..........
S.scrofa_d   S.HE.F..N. V.....MALV ..ML.Q.... .......AA. N....HQFYT G.....R..E

yak          ACLRLEEGLR GAPLLKEDSS LAVRKYFHRL TLYLQEKRHS PCAWEVVRAE VMRTFSSSTN
Bos_taurus   ...TQ..... .......... .......... .......... .......... ...A......
Ovis_aries   ....Q..... .......... .....H...V .......G.. .......... .L.A......
S.scrofa_d   ..VMQ.A..E .T...E...I R......... .......SY. .....I.... ...S....R.

yak          LQESFRRKD-
Bos_taurus   .........-
Ovis_aries   ...R.....-
S.scrofa_d   ..DRL.K.E-
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Fig. 1: Comparison of the amino acid sequences of the IFN-α1 from yak and those from cow (GenBank accession No. A10045), porcine (Q09JG9), ovine (HM187765), sequences were analyzed with the alignment tool MEGA 4.0 Software; the identity of amino acid residues

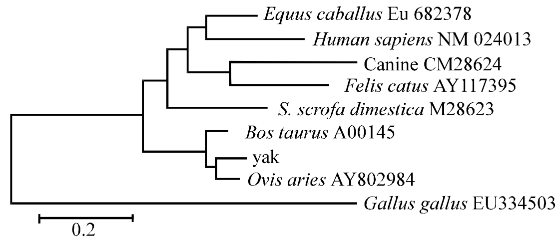

Fig. 2: Phylogenetic analysis of the yak IFN-α1 sequences and other species. Neighbor-joining trees were generated from homologies of IFN-α1 sequences and the confidence of the branching order was verified by making 1000 bootstrap replicates using the CLUSTALW program. The tree was viewed and converted to graphic format with TREEVIEW

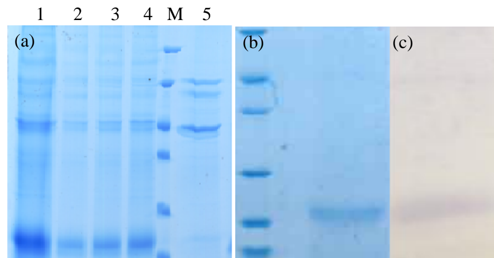

Fig. 3: a) SDS-PAGE analysis of yak IFN-α1 expressed in *P. pastoris*. Lane 1-4 expression product of different pPIC9K-IFN-α1/GS115 recombinant transformant from *P. pastoris* by methanol induced, Lane M: Maker of protein molecular (116, 66, 45, 35, 25, 18 and 14 kDa). Lane 5, pPIC9k/GS115 recombinant strain expression from *P. pastoris* by methanol induction. b) SDS-PAGE analysis of purified yak protein by sephadex G-100. c) Western blot analysis of yak IFN-α1 protein from *P. pastoris*. Lane M: contains the molecular weight markers, indicated in kilodaltons

Expression of yIFN-a1 in *P. pastoris*: In the initial screening, four positive transformants were identified by PCR with yIFN-a1 specific forward primer and 3'AOX1 primer. These clones were selected for protein expression trial. A protein band with the molecular weight of 20 kDa was detected on 12% SDS-PAGE from the induced GS115 pPIC9K-yIFN-a1 transformant (Fig. 3a). A high level of expression was obtained, the yields was up to 1.5 g L^{-1}. The secreted protein was purified from the culture medium by using gel chromatography (Fig. 3b). A specific band with the molecular weight of 20 kDa was detected after the purified yIFN-a1 was used as the antigen for immunoblotting with anti-human IFN-α1 antibody (BD company, American) (Fig. 3c).

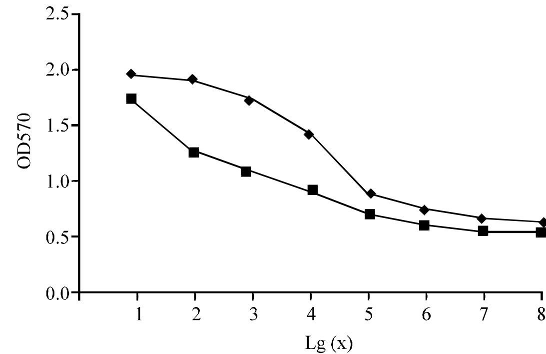

Fig. 4: Protection effect of hIFN-a1 standard and yak IFN-α1 on BHK cell. Antiviral activity of the yak IFN-α1 protein from BHK cells was incubated in the presence of the indicated dilutions of yak IFN-α1 for 24 h and subsequently infected with VSV. The cells were incubated 20 h following infection and CPE was determined by staining the cells and measuring the absorbance at 570 nm. Symbols are as follows: hIFN-α1 (■), yak IFN-α1 (♦) x represent sample dilution folds. Two similar curve both human IFN-α1 and yak IFN-α1 protein were drawn according to log (x) value as x-axis and OD570 value in different dilution folds as y-axis. Researchers draw a equation y = -0.3342 x +2.646 (R^2 = 0.938) by linear fitting according to the four point near the value of standard human IFN-α1 ED50 and draw a other equation y = -0.1808+1.626 (R^2 = 0.9982) according to the four point near the value of yak IFN-α1 ED50. The bioactivity was calculated according to the date supplied with equation

Anti-virul activity of recombinant IFN-α1: The antiviral activity of purified yIFN-a1 protein was determined *in vitro* with the crystal violet stain assay. A significant reduction in the VSV-induced Cytopathic Effect (CPE) was observed and the Optical Density that (OD) was measured. The serially diluted standard human IFN-α1 and the yIFN-a1 protein exhibited antiviral activity in BHK cell line against VSV and displayed in Fig. 4. The bioactivity was evaluated according to the date supplied by equation (Table 1) (Zhang *et al*., 2009). The yIFN-a1 titer (units per milliliter ($U\,mL^{-1}$)) was calculated according the following equation:

$$\text{Yak bioactivity } (IU\,mL^{-1}) = \frac{\text{PStandard} \times \dfrac{\text{DSample} \times \text{ESample}}{}}{\text{DStandard} \times \text{EStandard}}$$

Where:

Pr	=	Standard biocativity ($IU\,mL^{-1}$)
Dsample	=	Examine sample predilution folds

Table 1: The bioactivity of IFN-α1 was calcuated according to the date supplied by equation

Items	Human IFN-α1	Yak IFN-α1
ED50	1.285	1.285
K	-0.3342	-0.1802
b	2.646	1.626
D	50	3
Titer	2.5×10^7	1.16×10^5
E	10000	77.62

Esample = Dilution folds median Effective Dose (ED50) of examine sample equal to standard sample
Dstandard = Predilution folds of Standard sample
Estandard = Dilution folds of Standard sample in 50% CPE

The antiviral activity of the purified yIFN-a1 was calculated, about 1.16×10^5 IU mg^{-1}.

DISCUSSION

For several years, interferon a1 has been studied for treatment of various viral diseases such as hepatic fibrosis caused by hepatitis B, herpes simplex virus keratitis and bovine respiratory diseases in calves (Philip *et al.*, 2001; Jin, 1992). IFN-α1 plays an important role in host defense against viruses infection, specially human IFN-α1. Although, the information about IFN-α1 from some species can be available, there was no reports on yak IFN- α1. In this present study, researchers reported cloning and characterization of IFN-α1 from yak, the phylogenetic analysis of IFN-α1 among different species indicated that yak IFN-α1 is evolutionally more closer to IFN-α1 from cow and sheep than other species.

The cDNA prepared from total RNA isolated from ConA stimulated yak PBMCs, revealed the presence of IFN-α1 mRNA in PCR, a fragment size of 570 bp was obtained in RT-PCR amplification. To expressed the recombinant IFN-α1 in *Pichia pastoris*, the sequences without signal peptide were cloned into pPIC9K eukaryote expression vetor. *P. pastoris* is a methylotropic yeast strain which possesses prokaryote features including easy manipulation, clear genetic background and low cost. This yeast system also exhibits eukaryote features including folding and secretion mechanisms by which proteolysis, folding and posttranslational modification process such as glycosylation can be properly processed (Philip *et al.*, 2001). *P. pastoris* expression system uses Alcohol Oxidase (AOX) as a promoter which is strictly activated by methanol. AOX I initiates the Alpha Mating Factor (AMF) signal peptide transcription, leading to synthesize and secrete target proteins (Cregg *et al.*, 1993). Researchers were able to efficiently produce recombinant yak protein in relatively high yields with greater biological activity. The observed molecular weight of recombinant IFN-α1 in the present was 20 kDa as agreement with the expected molecular.

The IFN-α1 cytokine belong to multi-subtype family with broad spectrum of antiviral and immunomodulatory effects. Many study results demonstrated the antiviral activity of IFN-α subtypes is different *in vitro* and *in vivo* (Yeow *et al.*, 1998). In mouse, the IFN-α1 subtype appeared to be more protective against Murine CMV (MCMV) than the IFN-α4 or the IFN-α9 subtypes *in vivo*. Similar differences in antiviral activity of human IFN-α subtypes have been described (Koyama *et al.*, 2006; Larrea *et al.*, 2004; Schanen *et al.*, 2006). Researchersstudied the antiviral activity of yak IFN-α1 against VSV. The biological activity of yIFN-a1 protein showed that reduction of the VSV-induced CPE *in vitro*. The results showed that recombinant IFN-α1 has a specific anti-virus activity similar to the commercially available recombinant human IFN-α1.

CONCLUSION

In this study, this is the first report of the cloning and characterization of the yak IFN- α1 with facilitates the way for studies of the biological and therapeutic effects of this cytokine. Recombinant yak IFN-α1 protein was efficiently produced using *P. pastoris* and the protein posses anti-virus biological activity. *P. pastoris* has become an important host organism for the production of foreign proteins (Daly and Hearn, 2005; Macauley-Patrick *et al.*, 2005) and is also a powerful tool for obtaining proteins with activity on a large-scale (Cereghino and Cregg, 2000). The protein with good activity come from people and animals could be obtained by optimizing the conditions for yeast fermentation, the fementation process it is help to better prevention and treatment of corresponding diseases. Therefore, expressed product of yak IFN-α1 from *P. pastoris* system will provide basis for research and application in the future.

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