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Porcine IFN-β is Biologically Active and Secreted in the Yeast *Pichia pastoris* Expression System

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Abstract: Porcine Interferon (PoIFN) is a multifunctional secretory protein produced by animal immune cells. IFN signaling activates host cells resulting in anti-viral and -tumor immune regulation in addition to other biological functions. Here, the coding region of PoIFN-β was amplified from porcine genomic DNA by Polymerase Chain Reaction (PCR) and was cloned into the yeast *Pichia pastoris* expression system. This resulted in high levels of gene expression. Furthermore, expression of PoIFN-β was successfully detected by Western-blot. The relative molecular weight of the product was 25 and 27 kDa. The recombinant PoIFN-β exhibited cross-species antiviral activity in Marc-145 and BHK-21 mammalian cell lines. In summary, the results demonstrate that recombinant PoIFN-β cloned into *P. pastoris* is expressed, secreted and biologically active in mammalian cells.

Key words: Porcine interferon-β (PoIFN-β), *Pichia pastoris*, expression, activity, cells, biologically active

INTRODUCTION

Porcine Interferon (PoIFN) is a kind of secretive protein with multifunction produced by immune cells. Over the past several decades, a variety of interferon genes have been cloned from a number of species including human and porcine (Roberts *et al*., 1998; Chinsangaram *et al*., 2001; Matzener *et al*., 2009). IFN has anti-virus and -tumor immune regulatory activities (Lefevre *et al*., 1990; Weingartl and Derbyshire, 1991; Sen and Lengyel, 1992; Kishko and Vasylenko, 1999; Cereghino and Cregg, 2000; Samuel, 2001; Peng *et al*., 2004; Inan *et al*., 2006). Interferon-β (IFN-β) is primarily produced by fibroblasts and specific functions of the protein have been confirmed by numerous reserchers (Goodsell, 2001; Biernacki *et al*., 2005; Hidmark *et al*., 2005). IFN-β mediated activities occur through many different mechanisms such as stimulation of antiviral protein production, activation of natural killer cells and increasing the expression of MHC-II on antigen presenting cells (Thomson, 1994; Al-Khatib *et al*., 2004). Due to the unique properties of IFN-β (Adams *et al*., 2004; Moraes *et al*., 2007), it has been used experimentally along with vaccines to reinforce immune function or alone to improve immune reaction in pigs. Clearly, IFN-β has a broad and significant potential for use in the swine industry.

Cloning of the porcine *IFN-β* gene was 1st reported by Artursson *et al*. (1992). This was accomplished through amplification of the porcine *IFN-β* gene with no introns derived from the single known copy in the porcine genome. Chun *et al*. (2000) reported for the 1st time on the nucleotide sequence of the porcine *IFN-β* gene and comprised of 668 nucleotides, yielding a protein of 186 amino acids with a predicted molecular weight of 21.8 kDa. Subsequently, a large number of researches showed that the interferon is a highly specific, fast-acting and a broadly active anti-viral substance with both defensive and inhibitory activities (Wang *et al*., 2003, 2004a; Peng *et al*., 2005). The reported data showed that IFN-β had strong anti-viral effect against porcine pseudorabies, foot and mouth disease, swine fever, porcine reproductive and respiratory syndrome, swine influenza and porcine epidemic diarrhea viruses (Derbyshire, 1989; Brown *et al*., 2000; Chinsangaram *et al*., 2001; Rui-Bing *et al*., 2004; Cao *et al*., 2006; Overend *et al*., 2007; Yao *et al*., 2007a). The *Pichia pastoris* expression system is a powerful tool for the production of recombinant proteins that has been widely used for such purposes (Clare *et al*., 1991; Cregg *et al*., 1993; Cereghino and Cregg, 2000; Cregg *et al*., 2000a, b; Cereghino *et al*., 2002; Boettner *et al*., 2002; Macauley-Patrick *et al*., 2005; Jahic *et al*., 2006; Yao *et al*., 2007b; Li *et al*., 2007; Ghosalkar *et al*., 2008a). Mammalian proteins produced in

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yeast have been shown to be more similar to the natural material than that expressed in *Escherichia coli* (Romanos, 1995; Sreekrishna *et al*., 1997; Hong *et al*., 2002). The expression system used in the current study was based on the potent and tightly regulated methanol-inducible Alcohol Oxidase (AOX1) promoter (Tschopp *et al*., 1987). For these described reasons, Pichia is an ideal system for in ducible expression of heterologous proteins, capable of retaining biological activity (Higgins, 2001). In addition, the yeast expression system has the advantages rapid growth properties while requiring relatively little nutrients from inexpensive and easy-to-prepare culture medium.

The high-density fermentation technology further enhance the expression (Cereghino and Cregg, 2000; Kobayashi *et al*., 2000; Hong *et al*., 2002; Daly and Hearn, 2005; Richter *et al*., 2006; Ghosalkar *et al*., 2008b). Chinsangaram *et al*. (2001) reported that the *IFN-β* gene has a low expression in *E. coli*. Herein, researchers constructed a recombinant IFN-β *P. pastoris* cell line exhibiting high levels of protein secretion with anti-viral activities up to 5×10^4 U mL^{-1} in Marc-145 cells with PRRSV challenges.

MATERIALS AND METHODS

Cloning of PoIFN-β: Genomic DNA was isolated from pork liver and used to amplified the *PoIFN-β* gene by Polymerase Chain Reaction (PCR) employing the upstream 5'-CGGAATTCGAT GAGCTATGATGTGCTTC-3' and downstream primers 5'-GGGGTACCTCGTTCCGGAGGT AATCTG-3' which including EcoRI and KpnI restriction sites, respectively. Primers were designed according to the PoIFN-β sequence registered in NCBI GenBank (S41178) with the addition of a stop codon. For PCR reactions, 1 mg of porcine genomic DNA was used in a reaction volume of 100 mL (EX Buffer 2.5 μL, 2 mM $MgCl_2$, pH 8.3, 200 mM dNTPs, DNA IFN-β 3.0 μL, H_2O 16.5 μL) containing 100 pmol of each oligonucleotide primer and 2.5 U of *Thermus aquaticus* DNA polymerase. PCR was conducted in for 30 cycles of 30 sec at 94°C (4 min for the 1st cycle), 30 sec at 58.5°C and 30 sec at 72°C (7 min for the last cycle). The amplified fragment of 495 bp was detected using agarose gel electrophoresis. This fragment was linked with the vector pMD18-T and then used to transform *E. coli*.

The positive clone called pMD-PoIFN-β was obtained. Then the target gene and pPICZαC were both digested with EcoRI and KpnI. The fragments were then sub-cloned into the EcoRI and KpnI double-digestion site of the pPICZαC vector. The positive plasmid was designated as pPICZαC-PoIFN-β (Fig. 1).

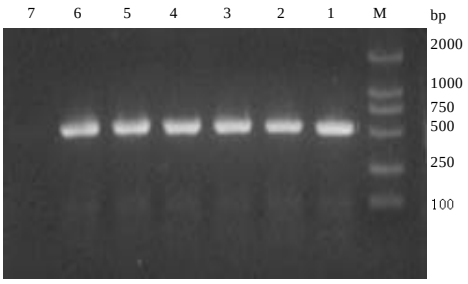

Fig. 1: Electrophoreses of Polymerase Chain Reaction (PCR) products of *PoIFN-β* gene. Lane M is 2000 bp DNA Marker ladder. Lanes 1-6 are PCR products samples of the *PoIFN-β* gene. Lane 7 is negative control

Expression of the rPoIFN-β in *P. pastoris*: The expression plasmid pPICZαC-PoIFN-β was linearized by with SacI and used to transfect the *P. pastoris* X-33 strain by electroporation. The transformants were identified by PCR using PoIFN-β and AOX1 specific primers. Dozens of recombinant colonies grew up on the $YPDS^+$ Zeocin™ (100 ug mL^{-1}) selective culture medium. During the production phase, the Mut^+ transformants were grown in Erlenmeyer flasks with 20 mL of BMGY solid medium (10 g yeast extract, 20 g tryptone, 13.4 g yeast nitrogen base, 4×10^{-5}D-biotin, 1% glycerol and 100 mmol L^{-1} phosphate buffer, pH 6.0 for 1 L) at 30°C until obtaining an OD_{600} nm of 2-6 was obtained. Then the cells were harvested and incubated in BMMY (10 g yeast extract, 20 g tryptone, 13.4 g yeast nitrogen base, 4×10^{-5} D-biotin, 0.5% Methanol and 100 mmol L^{-1} phosphate buffer, pH 6.0 for 1 L) at 30°C for 3-4 days with 1% methanol to induce expression. The expression of PoIFN-β, rPoIFN-β was determined by densitometric scanning of Coomassie brilliant blue stained protein bands fractionated by SDS-PAGE.

Analysis by Western-blot: The expressed supernatant was concentrated and identified by 12% Polyacrilamide Gel Electrophoresis in the presence of Sodium Dodecyl Sulfate (SDS-PAGE) and Western-blot using mouse-anti-swine beta interferon monoclonal antibodies and goat-anti-mouse-IgG-HRP.

Determination of *in vitro* antiviral activity of rPoIFN-β: Antiviral units per mL in a standard cytopathic effect assay was as previously reported. Briefly, dilutions of the recombinant PoIFN-β were incubated for 16-18 h at 37°C, 5% CO_2 with BHK-21 and Marc-145 cells. The capacity of for preventing the cytopathic effect on BHK-21 cells challenged with VSV was used to determine PoIFN-β

antiviral activity in the culture media of the methanol-induced transformants. The cells were primed for 18-20 h with dilutions of rPoIFN-β or control supernatants. The cells were then infected with VSV or PRRSV (GD08 strain) for 1.5-2.0 h, washed and replenished after infection. For detection of CPE, the cells were incubated for up to 7 days post-inoculation. One antiviral units were calculated as the dose capable of protecting 50% of wells with confluent cells from specific viral challenge. Antiviral activity was expressed in International Units (IU) as the reciprocal of the last dilution capable of preventing virus-induced cytopathic effect.

RESULTS AND DISCUSSION

Cloning of PoIFN-β in *P. pastoris*: The PoIFN-β coding sequence was obtained from pork liver by PCR as a fragment of 512 bp (Fig. 1) and was then cloned into the EcoR I and Kpn I site of the pPICZαC expression vector. The PoIFN-β gene was incorporated into the pPICZαC-PoIFN-β plasmid in the *P. pastoris* (Fig. 2).

Expression of the PoIFN-β gene in *P. pastoris*: The exoression of recombinant PoIFN-β was analyzed by SDS-PAGE (Fig. 3). As shown in Fig. 3, proteins were observed as having molecular weights of approximately 25 and 27 kDa.

These bands were shown by Western-blot using Mouse anti-swine IFN-β monoclonal antibody to be IFN-β with 2 species identified possessing molecular weights of 25 and 27 kDa as demonstrated by Western-blot analysis (Fig. 4). The appearance of 2 bands of similar molecular weights reacting with the anti-IFN-β monoclonal antibody suggest the presence of a species exhibiting alternative glycosylation.

Anti-viral activity of the PoIFN-β expressed by *P. pastoris* transformants: PoIFN-β ant-iviral activity was characterized by measuring the inhibition of the viral cytopathic effect in two different animal cells. The titers obtained with PoIFN-β were expressed as the highest dilution of IFN-β that protected 50% BHK-21 against specific viral challenge. The activity of the recombinant protein was attained up to 6×10^5 U mL^{-1} in BHK-21 with VSV and 5×10^4 U mL^{-1} in Marc-145 with PRRSV.

IFN-β secreted by the *P. pastoris* X33 strain was detected by Western-blot as described before for IFN-α and IFN-β. The results of Western-blotting indicated that the two proteins (25 and 27 kDa) to which the anti-poIFN-β monoclonal antibody reacted with are of PoIFN-β. However, given slight increase of the observed

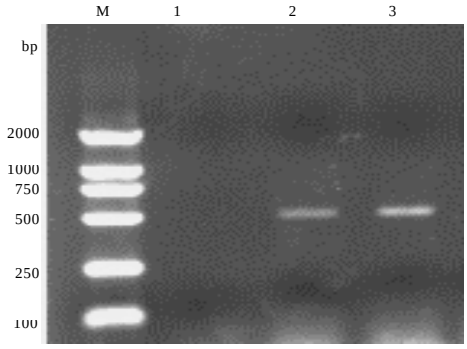

Fig. 2: Electrophoreses of Polymerase Chain Reaction (PCR) products of *PoIFN-β* gene isolated from recombinant pPICZαC-PoIFN-β. Lane M is 2000 bp DNA marker ladder. Lanes 1 is PCR product from pPICZαC transformed yeast. Lanes 2-3 are PCR products of pPICZαC-PoIFN-β transformed yeast

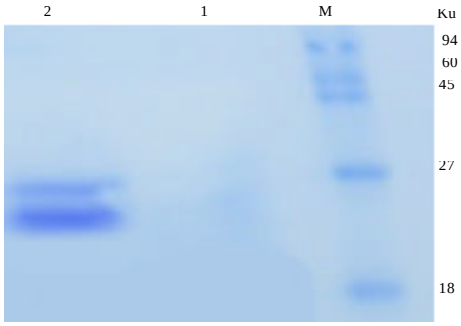

Fig. 3: SDS-PAGE of PoIFN-β protein by expressing in *P. pastoris*. Lane M is protein marker. Lane 1 is pPICZαC protein expressed in yeast. Lane 2 is pPICZαC-PoPoIFN-β protein expressed in yeast

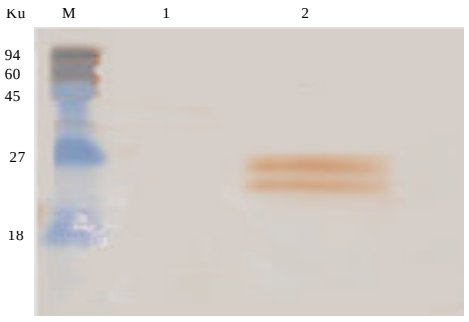

Fig. 4: Western-blot analysis of PoIFN-β protein. Lane M is protein marker. Lane 1 is pPICZαC protein. Lane 2 is pPICZαC-PoPoIFN-β protein

versus deduced theoretical molecular weight of PoIFN-β, two major expressed proteins are believed to

be the result of alternative glycosylation of the protein (Huang *et al*., 2005; Cao *et al*., 2006). Several approaches have been described to select multi-copy *P. pastoris* strains naturally existing within a transformed cell population (Cregg *et al*., 1993; Hong-li *et al*., 2004; Wang *et al*., 2004b; Hong-miao *et al*., 2005; Hu *et al*., 2007). Improved recombinant protein expression yields were observed for these multi-copy clones. Furthermore, reduction of the medium volume improved the yield of the expressed protein.

Based on the results, there was employed high cell density fermentation with this strain, allowing accumulation of the product in the medium for 96 h under methanol-inducible conditions.

In contrast with several other recombinant proteins secreted from *P. pastoris*, PoIFN-β was exceptionally stable in the bioreactor medium which was supported by increased expression levels being reached following prolonged fermentation.

Reports indicated that the combination of type I and II IFNs act synergistically to inhibit FMDV replication *in vitro* and *in vivo* (Buddaert *et al*., 1998; McRae *et al*., 2000; Adams *et al*., 2004; Moraes *et al*., 2007) and that IFN-α production clearly is beneficial for suppressing PRRSV replication as it has been demonstrated to efficiently block replication when present during infection (Delputte *et al*., 2007; Loving *et al*., 2007). IFN-β can also protect macrophages against PRRSV infection (Overend *et al*., 2007). The PoIFN-β produced by the *P. pastoris* showed exceptional antiviral activity in different animal cell lines as is evidence by the observed concentration of 6×10^5 U L^{-1}.

CONCLUSION

In this study, the biologically active PoIFN-β was secreted at high levels in *P. pastoris*. These findings demonstrate a relatively simple and cost-effective method for production of PoIFN-β to control a wide variety of highly infectious and economically important porcine viral diseases. However, further studies are required to test the efficacy of this recombinant IFN-β *in vivo*.

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