



Molecular characterization of IFN-T expressed in buffalo embryonic trophoblasts and expression of recombinant BuIFN-T1a2 and BuIFN-T8 isoforms in *E. coli*

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ABSTRACT

Interferon tau (IFN-T) acts as a signaling molecule for maternal recognition of pregnancy (MRP) in ruminants. Aim of the present study was to identify various Buffalo Interferon tau (BuIFN-T) transcripts in buffalo trophoblast, phylogenetic comparison of these sequences with known mRNA sequences of buffalo, bovine, caprine and ovine and to express and purify the recombinant BuIFN-T (rBuIFN-T) isoforms. Following RNA extraction from trophoblast cells, RT-PCR was performed using *Ifn-t* gene specific primers. 13 distinct cDNA variants encoding eight different BuIFN-T proteins were identified. BuIFN-T1a2 and BuIFN-T8 were expressed in prokaryotic expression system at 37 °C, 25 °C and 16 °C with 1 mM IPTG for 12 h and the recombinant proteins expressed at 16 °C were partially purified by Immobilised Metal Affinity Chromatography (IMAC). BuIFN-T isoforms have greater nucleotide and amino acid homology with caprine (98–100%, 96–100%), ovine (94–97%, 90–95%) and bovine (89.6–90.6%, 82–86%). These novel BuIFN-T isoforms contained pronounced nucleotide and amino acid sequence identity with one another (99.1–99.8%, 98–99%) but moderate sequence identity with previously identified buffalo IFN-T (90–92%, 82–86%). Solubility of expressed recombinant isoforms (rBuIFN-T1a2 and rBuIFN-T8) was highest at 16 °C. In conclusion, 13 distinct *Ifn-t* gene variants exist in trophoblast of *in vitro* developed buffalo blastocysts that encode eight different proteins. rBuIFN-T1a2 and rBuIFN-T8 were successfully expressed in soluble form in *Escherichia coli* expression system at 16 °C with 1 mM IPTG and the resulting recombinant proteins were partially purified by IMAC.

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1. Introduction

MRP and subsequent establishment of embryo in uterus are two vital events for fruitful pregnancy. This needs maintenance of corpus luteum (CL) beyond normal estrous cycle which depends on signals received from the developing embryo. Interferon tau (IFN-T) is one such signaling molecule which is synthesized in trophoblast cells of blastocyst and then secreted to act on uterine epithelial cells for further signaling resulting into diverse physiological actions like implantation, maternal recognition of pregnancy, prevention of immune rejection etc.

IFN-T was initially termed as trophoblastin [1] or trophoblast

protein-1 [2,3]. The first report of IFN-T came in ovine [1] then it was discovered in almost all the ungulate species like cattle, goat, buffalo, red deer etc. [4,5]. IFN-T is exclusively expressed in trophoblast cells of blastocyst and its expression is temporal till the implantation of blastocyst. *Ifn-t* belongs to Type I IFNs [6] and are encoded by multiple genes [7]. Conceptus-derived IFNs are structurally distinct from other Type I IFNs though they possess many activities of Type I IFNs, such as antiviral, immunomodulatory and antiproliferative capabilities. They are collectively referred as IFN-T [8]. Unlike most Type I IFNs, IFN-T expression is not induced by viral or bacterial pathogens [9] and is expressed constitutively by the trophoblast of blastocyst till the attachment of elongated conceptus to uterine wall [10–13].

IFN-T inhibits regression of CL by suppressing endometrial prostaglandin F_{2α} (PGF_{2α}) release [14,15]. IFN-T prevents oxytocin receptor expression in endometrial epithelium, thereby preventing

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oxytocin from stimulating synthesis and release of PGF 2α [16]. It has been estimated that there may be as many as 18 *Ifn-t* genes in cattle, all of them clustered within or in close proximity to the genetic locus of Type I *Ifn* genes [17]. Presently, 18 distinct polymorphic ovine and 18 bovine alleles have been identified [18]. Multiple ovine and bovine *Ifn-t* genes are transcribed during early pregnancy which encodes proteins that can possess different biological activities [7,11].

In buffalo, there is report of only one isoform of *Ifn-t* (ACCESSION NO: AY535404). The present work has been done with the objective to identify various *Ifn-t* transcripts in the trophoblastic cells of *in vitro* cultured buffalo blastocyst, to compare these nucleotide sequences phylogenetically with the reported mRNA sequences of buffalo, cattle, sheep and goat, to know the relatively predominant isoform expressed at mRNA level and to clone and express it.

2. Material and methods

2.1. *In vitro* embryo production

Buffalo ovaries collection, oocyte aspiration, *in vitro* maturation, *in vitro* fertilisation and *in vitro* embryo production were performed as described earlier [19,20].

2.2. Isolation of primary trophoblastic cells from hatched blastocysts

Each of the hatched blastocysts were seeded separately on 4 well plate (Nunc, Denmark) containing standard culture medium (DMEM/F12 supplemented with 10% FBS, 50 μ g/ml gentamycin) at 38.5 °C under 5% CO $_2$. The spent medium was replaced with fresh cultured medium in an interval of 48 h till sufficient outgrowths (10 days) from hatched blastocyst were seen. Inner cell mass was removed mechanically and the outgrowths of the trophoblastic cells were collected by trypsinisation, 0.25% trypsin for 3 min at room temperature.

2.3. RNA extraction, RT-PCR, cDNA cloning and sequence analysis

In order to avoid the variation in the gene in population, the trophoblastic outgrowths of single hatched blastocysts were used in the whole experiment. Total cellular (tc) RNA was extracted from trophoblastic tissue using the RNeasy mini kit (Qiagen Corp., Carlsbad, CA) according to the manufacturer's instructions. RNA preparations were treated with RNase-free DNase enzyme (Qiagen Corp., Madison, WI) for 1–2 min at room temp to remove genomic DNA contamination.

2.4. Primer designing

Ifn-t sequences of different isoforms in cattle were retrieved from NCBI Genbank (Accession No: AF238611.1, AF238612.1, AF238613.1) and a consensus sequence was determined using clustalW multiple alignment program of DNASTar. The consensus sequence was used to design primers by Primer3 software (primer3.ut.ee/) to amplify full length *Ifn-t* in buffalo.

2.5. Synthesis of cDNA, PCR and sequence analysis

Two micrograms of tcRNA was incubated at 65 °C for 5 min, then reverse transcribed with M-MuLV reverse transcriptase (#K1621, Fermentas Corp, USA), oligo(dT) primer, and 10 mM each of dNTP mix at 42 °C for 60 min. PCR amplification of *Ifn-t* genes were performed with high fidelity dream taq DNA Polymerase (Fermentas Corp, USA) and *Ifn-t* specific primers in 20 μ l reaction

volume. Briefly, reaction mix contained 10 μ l dream taq™ green PCR master mix (Fermentas Corp, USA), 8 μ l NFW, 0.5 μ l of each primer and 1 μ l cDNA. The cyclic conditions used for PCR were: initial denaturation at 94 °C for 3 min; 35 cycles of - 94 °C for 30 s; 62 °C for 30s and 72 °C for 1 min; and final extension at 72 °C for 10 min. The sequence of primers used in this study was- forward primer: 5'-AACCTACTGAAGGTTACCCAGA-3' and reverse primer: 5'-TGAGTGACGAAGGTGATGTGGCA-3'. PCR product was purified on 1.5% agarose and ligated into the pJET1.2/blunt cloning vector (Fermentas Corp. USA) and transformed in TOP10 competent *E. coli* cells according to the manufacturer's instructions (Invitrogen Inc., Carlsbad, CA, USA) and plated on Luria Bertani Agar (LB) plates containing 50 μ g/ml ampicillin. Bacterial colonies were picked and propagated in 5 ml Luria Broth containing ampicillin at 37 °C overnight. Plasmid was isolated using the Nucleospin Plasmid Miniprep Kit (Genetix Biotech Asia, New Delhi). PCR amplification using IFN-T specific primers verified presence of Buffalo IFN-T gene. Total 30 clones were sequenced twice from two different labs (Eurofins MWG Operon, India; Xcelaris genomics, Ahmedabad), in both directions using vector primers and sequences were compiled. The identified novel buffalo *Ifn-t* variants and the coding sequences of bovine, ovine and caprine *Ifn-t* variants listed in Genbank, were compared through multiple alignment using ClustalW, BioEdit version 7.09. For phylogenetic analysis, a consensus tree was constructed using MEGA version 4 (<http://www.megasoftware.net/>) through the Maximum Parsimony method [21].

2.6. Construction of prokaryotic expression vector of BuIFN-T

TOP10 cells harbouring isoforms BuIFNT1a2 and BuIFNT8 in pJET vector were verified, and PCR amplified, without signal sequence, using 5'-end primers having NcoI restriction sites (5'-atcGCCATGGTGTACCTATCTCGGAGACTCATG-3') and 3'-end primer having XhoI restriction site (5'-atcGCTCGAGAGGTGAGTTCA-GATTCACCCAT-3'). The cyclic conditions used for PCR amplification were same as described earlier. Amplicons were cloned in pET-28b vector after double restriction digestion and transformed in TOP10 cells. Recombinant colonies having correct ORF were used to transform BL21 (DE3) *E. coli* cells.

2.7. Expression of recombinant buffalo IFN-T (rBuIFN-T) isoforms

The colonies containing recombinant plasmid pET-28b-BuIFN-T (the positive clones) were grown to 0.6 OD at 600 nm and were initially induced with 1 mM IPTG at 37 °C for 4 h. To improve solubility, expression was tried at lower temperature- 25 °C and 16 °C for overnight and 22 h, respectively with 0.5 mM IPTG. Soluble proteins from respective cell lysates were extracted using Q proteome Bacterial Protein prep kit (Qiagen, USA) according to manufacturer's instruction. The soluble protein and insoluble fraction from pellet were subjected to SDS-PAGE and western blot to analyze expression of recombinant proteins.

2.8. Purification of rBuIFN-T isoforms using His-tag affinity column

For purification of soluble rBuIFN-T1a2 and rBuIFN-T8, single colony of BL-21 DE3 cells harbouring BuIFN-T1-pET28b + & BuIFN-T8-pET28b + expression constructs were cultured in 2 L LB broth for 12 h at 16 °C with 50 μ g/ml ampicillin. Cells were harvested at 6000 g for 20 min and resuspended in His binding buffer (0.3 M NaCl, 10 mM imidazole in 50 mM phosphate buffer) and sonicated (conditions- 40 amplitude, 5 s pulse, 5 s on/off for 30 min). Soluble and insoluble fractions were then separated at 15,000 g for 30 min at 4 °C and the cleared lysates were used for purification. The supernatant of each recombinant protein, was loaded into 1 ml

HisTrap HP cartridge (GE Healthcare) for purification of His-tag eluted rBulFN-T protein through IMAC. After equilibration of column with His binding buffer, protein was allowed to bind at a flow rate of 1.0 ml/min and the bound protein was washed with wash buffer (50 mM sodium phosphate buffer, 0.3 M NaCl, 20 mM imidazole, pH 8.0). Adsorbed His-tag-BulFN-T was eluted with imidazole step wise gradient. A gradient of 0–30% was run in 50 ml of elution buffer (1 M imidazole, 50 mM sodium phosphate buffer, 0.3 M NaCl, pH 8.0). The purified fractions (i.e. the His elutes containing the recombinant fusion protein), washing fraction (i.e. fraction collected during washing with 20 mM imidazole wash buffer), unbound fractions along with cell pellet and cell lysate (positive control) were subjected to SDS-PAGE.

2.9. Western blot

The purified recombinant BulFN-T proteins- rBulFN-T1a2 and rBulFN-T8 were confirmed by western blot analysis. For western blot, the membrane was incubated with primary antibody, anti 6x-His Epitope Tag mice Antibody (Pierce, Thermo scientific), at 1:1000 dilutions and secondary antibody, horseradish peroxidase-conjugated Goat Anti-Mouse IgG (Abnova, Thermo scientific), at 1:2000 dilution.

2.10. Antiviral assay

The antiviral assay for the target protein was performed by using Madin-Darby bovine kidney cell line (MDBK) and Chandipura virus (CHPV). The MDBK cell monolayer for assay was developed as mentioned earlier [22]. Initially, to produce working stock, 1×10^8 plaque forming units (PFU) of CHPV was propagated in BHK-21 cells to attain approximately 1 multiplicity of infection (moi). Following development of virus induced cytopathic effects (CPE) in 80–90% cells of the monolayer, the PFU of CHPV to be used for antiviral assay was standardized as described earlier [22]. The Standard bovine IFN-T (My biosource, MBS836704) was diluted to 10-fold dilution and its activity against CHPV was estimated. To analyze the potency of test rBulFN-T1a2, it was diluted with broad range of dilutions i.e. 10-fold dilution, increment starting from 10^{-4} – 10^{-15} .

2.10.1. Assay protocol

4×10^4 MDBK cells suspension in MEM + 10% FCS was seeded in each well of 96-well flat bottom plate (Corning) and incubated at 37 °C in CO₂ incubator. After 4 h of seeding cells were fed with respective standard bovine IFNT and test rBulFN-T1a2 dilution, the virus control and cell control was fed with 100 µl of MEM + 10% FCS and incubated further. After 18 h of IFNT treatment, cells were infected with 0.1 ml of CHPV suspension containing 1×10^5 PFU and incubated further in CO₂ incubator at 37 °C. 30 h post virus infection (when virus control showed 100% CPE infection) the assay was terminated and the plates were washed and stained with 0.1% Amido Black stain as described earlier [22].

3. Results

3.1. Production of hatched blastocysts

In the present study, 182 cleaved zygotes were obtained and finally 15 blastocysts were hatched. These hatched blastocysts were cultured *in vitro* and expanded trophectodermal outgrowths were observed within 10 days of culture.

3.2. Sequence analysis, identification and nomenclature of various isoforms

The integrity of RNA isolated from trophectodermal cells was confirmed by two distinct bands corresponding to 18S and 28S rRNAs on 1.5% agarose gel (Fig. 1A). PCR amplification of *Ifnt* resulted in product of 694 bp length (Fig. 1B). Following cloning, sequence analysis yielded 13 different cDNA sequences (Fig. 1C). All these 13 cDNA sequences (defined as buffalo IFN-T: 1a1, 1a2, 1a3, 1b1, 1b2, 2a, 2b, 3, 4, 5, 6, 7 and 8 in supplementary file Fig. 1) represent an ORF (determined by DNASTAR and DNA to PROTEIN software) of 588 bp coding for 195 amino acids. Out of these 13 cDNA sequences, eight cDNA sequences upon predicted translation (by DNA star and DNA to PROTEIN software) encoded eight distinct proteins differing from each other by at least one amino acid (supplementary file Table 1). The cDNA sequences of these eight distinct proteins have been submitted to NCBI Genbank data base (Accession numbers: JX481982, JX481987, JX481989, JX481990, JX481991, JX481992, JX481993 and JX481994. Interestingly, the newly identified buffalo *Ifnt* isoforms contain pronounced nucleotide and amino acid sequence identity with each other (99.1–99.8%, 98–99%) but have only moderate sequence identity with reported buffalo *Ifnt* (90–92%, 82–86%) (supplementary file Figs. 1 and 2). Rest five sequences were differing at nucleotide level without any change in amino acid sequence (supplementary file Table 1).

Numeric values 1 to 8 represent distinct isoforms where difference in nucleotide sequence results in change in amino acids. Letters “a” or “b” following first numerical represents variants under specific isoform, which differ in nucleotide sequence without changing amino acid. The third numerical denotes different positional variants within same isoform (supplementary file Table 1).

The sequences of eight distinct isoforms of IFN-T in buffalo were compared to different isoforms of IFN-T in sheep, goat and cattle. The similarity among all these buffalo IFN-T isoforms and five bovine isoforms (Accession no: M31557, AF196320, AF196324, EU828780, EU828775), four sheep IFN-T (Accession no: M26386, NM_001123400.1, NM_001101735, NM_001123401) and five goat IFN-T (Accession no: AY357327, AY357329, AY357332, AY357335, AY357336) available in NCBI was 89.6–90.6%, 94.7–96.9% and 98.3–100%, respectively. Amino acid sequence analysis (supplementary file Fig. 2) and phylogenetic tree analysis (supplementary file Fig. 3) indicated high similarity of buffalo IFN-T sequences with

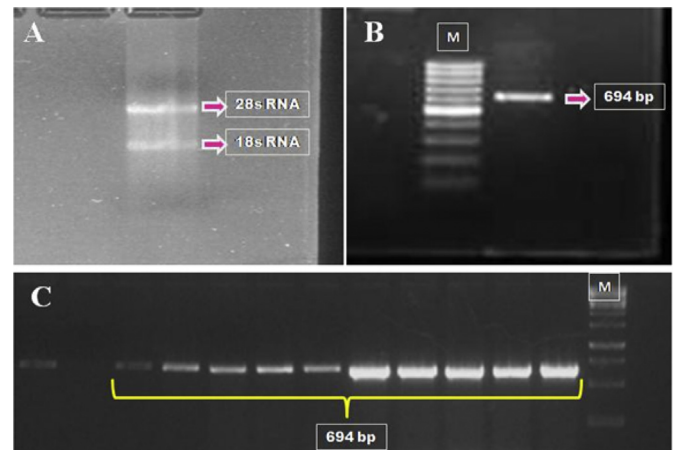


Fig. 1. RNA on 1.5% agarose gel revealing two distinct bands- 28s RNA and 18s RNA (A). PCR amplified product of BulFN-T (694 bp) (B). Amplified product of IFN-T from different recombinant clones (C).

cattle, goat, and sheep IFN-T. This suggests that the BulFN-T gene is closely related to IFN-T sequence of ruminants. However, among all ruminants, buffalo IFN-T group together with caprine IFN-T while ovine and bovine IFN-T make separate groups (supplementary file Fig. 3).

3.3. In silico analysis of predicted protein

The novel BulFN-T proteins are predicted to contain structural features indicative of IFN-T and other Type I IFNs, including five long helices (helices A–E) by PSIPRED v3.3 Secondary Structure Prediction server (supplementary file Figs. 1 and 4). In addition, the novel buffalo IFN-T proteins are expected to possess cysteine residues required for disulfide bridging (Cys24/Cys52 and Cys122/Cys162) (DISULFIND). Sequence analysis of predicted translation product of all isoforms reveals 23 amino acids as signal peptide at N terminal (Signal IP 4.1 Server). It seems these isoforms do not have O-linked glycosylation, but some of the buffalo IFN-T isoforms contain a putative N-linked glycosylation site (Asn78) (netOGlyc 3.1 and netNGlyc 3.1 prediction program). Similarly, Netphos 2.0 algorithm predicted three ser (25th, 28th and 74th position), five threonine (70th, 98th, 119th, 153rd and 163rd position) and two tyrosine (58th and 136th position) residues with potential phosphorylation sites.

3.4. Expression of recombinant BulFN-T isoforms

PCR products (Fig. 2A) of TOP10 cells, harbouring isoforms-BulFN-T1a2 and BulFN-T8 in the pJET cloning vector, were subcloned into pET28 vector. Transformed recombinant plasmid pET28b-BulFN-T1a2 and pET28b-BulFN-T8 in TOP10 cells were screened by colony PCR (Fig. 2B). The positive colonies were cross-checked by restriction digestion with Nco1 and Xho1 enzymes (Fig. 2C). The final positive clones transformed into *E. coli* BL21 (DE3) strain were analysed for correct ORF of the insert sequence (Fig. 2D). On induction at 37 °C with 1 mM IPTG for 4 h, these recombinant colonies expressed only insoluble aggregates of recombinant protein (rBulFN-T1a2 and rBulFN-T8) of about 20 kDa as confirmed by SDS PAGE and mass spectroscopy (Fig. 3A and supplementary file). To improve the solubility of recombinant proteins, cultured cells were induced for overnight at 25 °C and for 22 h at 16 °C with 0.5 mM IPTG. On the basis of band intensity in SDS-PAGE, it was observed that solubility of recombinant proteins improved at 16 °C (Fig. 3B).

3.5. Purification of recombinant BulFN-T isoforms

Purification of the rBulFN-T proteins through IMAC yielded two broad fractions- eluted fraction (during gradient of 300 mM Imidazole) and washing fraction (i.e. fraction collected during washing with 20 mM Imidazole wash buffer). SDS PAGE analysis (of each isoform) showed that both the fractions contained purified protein as major band (Fig. 3C and D). Interestingly, the washing fraction was having very low level of contaminating proteins as compared to the eluted fraction. Finally, the purified rBulFN-T1a2 and rBulFN-T8 were confirmed by western blotting (Fig. 4).

3.6. Antiviral activity of rBulFN-T protein

After 30 h of virus infection, the test rBulFN-T1a2 exhibited IFN activity corresponding to 10 IU/ml of standard bovine IFNT at 10^{-7} dilution. On estimation of total protein content in the lyophilized stock of test IFNT, it was found that 0.067×10^{-7} mg of rBulFN-T1a2 resulted in 50% reduction in CPE after 30 h of viral infection exposure. The specific antiviral activity exhibited by rBulFN-T1a2 was found to be 1.5×10^8 antiviral units/mg of protein parallel to the standard bovine IFN-T protein.

4. Discussion

Previously, variety of *Ifn-t* transcripts have been identified in caprine [23], ovine [7] and bovine [11] conceptus. From this study it can be said that at least eight distinct IFN-T proteins encoded by 13 *Ifn-t* variants are expressed in the buffalo conceptus. A single set of primers, derived from the consensus sequences of bovine and caprine, were used for PCR amplification of *Ifn-t* transcripts in buffalo trophectoderm. Due to high degree of homology between the buffalo IFN-T isoforms, single set of primers amplified all possible *Ifn-t* variant transcripts. But some isoforms having variation at the primer annealing site (because of degenerate sequences) might have been missed in this study. Owing to small size of *Ifn-t*, PCR generated errors were expected to be least, still the sequence obtained from two different labs were compared. The PCR sequence from two different labs were same and also the sequence readings of both the forward and reverse strands were same, so we can say with high confidence that there was no artefact in the sequence of the gene. Analysis of sequences gave rise to eight isoforms of buffalo IFN-T encoded by 13 *Ifn-t* cDNA variants. As per earlier reports *Ifn-t* evolved from *Ifn- ω* by gene duplication about the time ruminants diverged from other artiodactyls [4]. PCR amplified and sequenced *Ifn-t* using same primers on genomic DNA isolated from blood, following BLAST revealed *Ifn-t* to be closer to *Ifn- ω* . Hence,

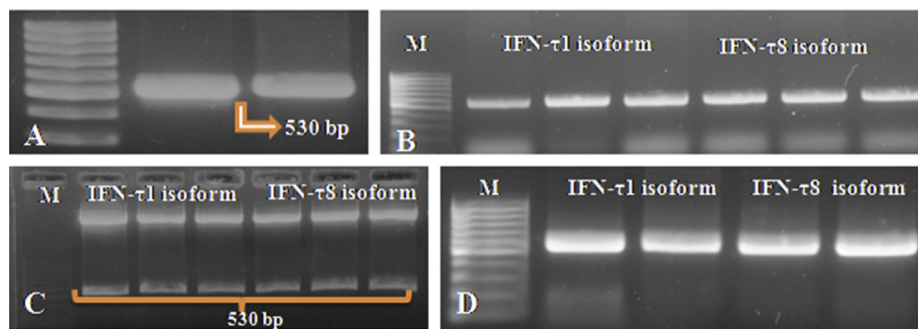


Fig. 2. PCR amplified products of both isoforms from cloning vector using F and R primers having Nco1 and Xho1 restriction sites, respectively (A). Screening of recombinant colonies for both isoforms from transformed top 10 cells: showing three positive clones each for BulFN-T1 and BulFN-T8 isoform (B). Cross checking of Top 10 cells positive clones by restriction digestion with Nco1 and Xho1 restriction enzymes: showing released product of 530 bp (C). Screening of recombinant colonies for both isoforms from transformed BL21 cells: showing two positive clones for BulFN-T1a2 and BulFN-T8 isoform, respectively (D).

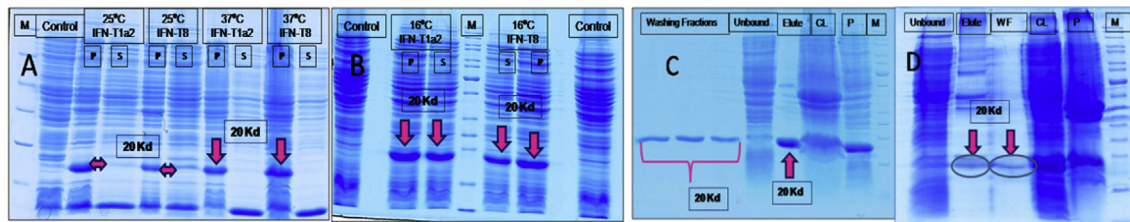


Fig. 3. SDS PAGE analyses of expressed and purified rBulFN-T1a2 and rBulFN-T8. Expression at 25 °C and 37 °C with 1 mM concentration of IPTG: lane M- Marker, lane 1- Uninduced (control), lane 2 and 3- BulFN-T1a2 at 25 °C in pellet and soluble fraction, respectively, lane 4 and 5- BulFN-T8 at 25 °C in pellet and soluble fraction, respectively, lane 6 and 7- BulFN-T1a2 at 37 °C in pellet and soluble fraction, respectively and lane 8 and 9- BulFN-T8 at 37 °C in pellet and soluble fraction, respectively (A). Expression at 16 °C with 1 mM IPTG: lane M- Marker, lane 1 and 6- Uninduced (control), lane 2 and 3- BulFN-T1a2 pellet and soluble fraction, respectively, lane 4 and 5- BulFN-T8 soluble and pellet fraction, respectively) (B). Purified rBulFN-T1a2: lane 1, 2 and 3- Washing fraction, lane 4- Unbound, lane 5- Eluted fraction, lane 6- Cell lysate, lane 7- Cell pellet, lane M- marker (C). Purified rBulFN-T8: lane 1- Unbound, lane 2- Eluted fraction, lane 3- Washing fraction, lane 4- Cell lysate, lane 5- Cell pellet, lane M- marker (D).

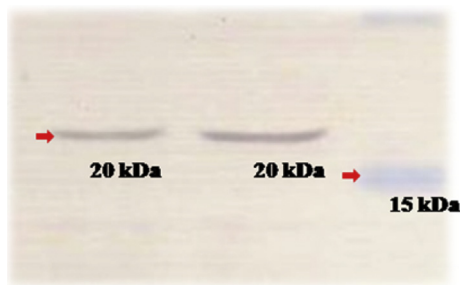


Fig. 4. Western blots of partially purified recombinant BulFN-T isoforms against anti-HIS antibody.

we advise a word of caution that probably because of similarity between *Ifn-t* and *Ifn- ω* , *Ifn- ω* genes are preferentially amplified over *Ifn-t*. Since, IFN-T expression is exclusive to conceptus, which leads to relatively more mRNA transcripts of *Ifn-t*, reverse transcriptase-PCR(RT-PCR) based approach is better than genomic (gDNA) based PCR.

Comparison of the nucleotide sequence of buffalo IFN-T isoforms with other species IFN-T isoforms followed by phylogenetic analysis revealed that BulFN-T gene sequence is closely related to goat (96–100%), sheep (90–95%) and cattle (82–86%) IFN-T sequences at amino acid level (supplementary file Fig. 2). Greater homology of buffalo IFN-T gene with caprine IFN-T gene may be correlated with similar reproductive physiology involved in embryo implantation, placentation and/or maternal recognition of pregnancy. A similar finding has been reported in IFN-T gene of *Bos frontalis*, where *B. frontalis* IFN-T gene was having more than 85% homology with IFN-T gene of other ruminants but in phylogenetic tree it made a clade with IFN-T gene of Red Deer [26]. Nevertheless, about 89–90% homology of IFN-T gene among all ruminants suggests that IFN-T gene is conserved across the species. Similar to the findings of Walker et al. (2009), in our study the overall similarity among the thirteen buffalo *Ifn-t* variants was quite high ranging between 99.1 and 99.8% at nucleotide level and 98–99% at amino acid level.

Historically, different isoforms of IFN-T have been named and symbolized differently in many species. In our study the different isoforms and variants have been named in a very simple way. The sequences varying only in their amino acid sequence were considered distinct isoforms which resulted in eight main groups of isoforms namely 1, 2, 3, 4, 5, 6, 7 and 8. Under each group, sequences which varied at nucleotides without altering the amino acids were considered as distinct variants of *Ifn-t* and denoted with isoform group number followed by letters a, b etc. Within such variants according to the position of variation these variants were

further distinguished as 1, 2, or 3. Sequence analysis of 30 clones resulting from two independent PCR products also revealed information about the relative abundance of various isoforms. The real time PCR based quantification of relative copy number would have been more informative. But as these isoforms were similar in sequences, distinct primers amplifying each isoform coding mRNA transcripts could not be designed. Thus in our study, based upon frequency of obtaining a particular type of sequence, it could be said that particular variant (i.e. IFN-T1a2) is probably transcribed in more copy and hence, represents more clones in the pool of randomly selected large number of recombinant colonies. IFN-T expression is exclusive to blastocyst and its expression increases from morula till hatched blastocyst and decreases post implantation [24]. Regarding role of different isoforms, it has been suggested that either specific isoform is unique to function during implantation [23] or to maintain enough molecules of IFN-T during implantation, IFN-T genes might have been duplicated [11,25]. Functional implication of different isoforms of IFN-T suggests that they differ in their biological potency- like all the isoforms have antiluteolytic function and antiviral activity but some have high antiviral activity while others have stronger antiluteolytic activity [7,10].

It is interesting to note that none of the buffalo IFN-T contain O-linked glycosylation. But majority of the buffalo IFN-T isoforms contain a potential N-linked glycosylation site at amino acid position 78 (Asn78 in buffalo IFN-T1, T3, T4, T5, T6, T7 and T8) where as bulFN-T2 isoform does not contain this site (Asp78). This suggests that both N-glycosylated and non-glycosylated forms of IFN-T proteins are produced by the buffalo conceptus, like caprine species [23]. In the ovine conceptus, some IFN-T isoforms contain Asn78 residue but none of these proteins are glycosylated [7,27]. In contrast to buffalo or goat, all known boIFN-T isoforms contain Asn78 and all IFN-T produced by bovine trophectoderm are glycosylated [11,27]. The functional importance of glycosylated moieties on IFN-T remains mystery. Non-glycosylated recombinant boIFN-T and native boIFN-T have similar biological activities [11,28]. In addition, glycosylated and non-glycosylated caprine IFN-T proteins purified from goat conceptuses also have identical biological activity [29]. Whether glycosylation enhances the functional lifespan of IFN-T activity in utero or not is unknown. Glycosylation of recombinant human IFN-B increased protein stability and biological longevity [30]. IFN-T is a secretory protein which after its translation is secreted out of cell and acts on uterine epithelial cells [31]. Initial 23 amino acids are predicted to be signal peptide which is conserved across ovine, caprine and bovine except BulFN-T8 in which leucine has been replaced by proline at 7th position. In our study, out of total 10 potential ser, thr and tyr phosphorylation sites, most of them seem to be present in 2/3rd C-terminal region. Molecules like IFN-T are involved in a cascade of signal transduction

reaction [31]. The catalytic activity of IFN-T may be attributed to the C-terminal region as was revealed by mutagenesis study [32].

In our study, from sequence analysis it was found that IFN-T1a2 was the major isoform and IFN-T8 was the isoform which diverged maximally from other isoforms. So the expression of these two isomers (IFN-T1a2 and IFN-T8) as recombinant protein was tried. Now, despite the advances in eukaryotic expression systems, *E. coli* remains the first host of choice for most researchers, as overproduction and purification of recombinant protein from *E. coli* is faster, cheaper and easier than from other organisms. For these reasons, of the 58 biopharmaceuticals products approved from 2006 to 2010, 17 are produced in *E. coli*, 32 are produced in mammalian cell lines (mainly Chinese hamster ovary; CHO) and four are produced in *Saccharomyces cerevisiae*, transgenic animals, or via direct synthesis [33]. However, the production of proteins to high yields remains a process of trial and error. Initially, as we tried to express the recombinant proteins at 37 °C with 1 mM IPTG for 4 h, it led to expression in the form of insoluble aggregates. To limit the *in vivo* aggregation of recombinant proteins cultivation of bacteria at reduced temperature, with reduced concentration of inducer [34,35] has been proved to be effective. So, we tried to express these proteins at 25 °C and 16 °C for overnight and 22 h, respectively with 0.5 mM IPTG. The solubility of these recombinant proteins increased significantly at 16 °C. Strong temperature dependent hydrophobic interactions that determine the aggregation reactions are favored at higher temperature [36]. At reduced temperature the heat shock proteases that are induced under over expression conditions are partially eliminated [37]. In *E. coli* the activity and expression of chaperones are increased at 30 °C [38,39]. All these factors partially explain increased stability and correct folding at low temperature. Our result was in contrast to a reported literature [40] which reported that bovine IFN-T expressed as inclusion bodies at low temperatures. Finally, the recombinant proteins expressed at 16 °C were purified by IMAC chromatography. Affinity chromatography by nickel–nitrilotriacetic acid (Ni-NTA) column resulted in two broader fractions, one was eluted fraction at 300 mM imidazole and other was the fraction collected during washing by 20 mM imidazole wash buffer. Recombinant protein was present as major band in both the fractions, as evident from SDS PAGE analysis (Fig. 3C and D). Surprisingly, number of contaminating bands in washing fraction was very less. Taking into account the greater yield of rBulIFN-T1a2, obtained in a single step purification and its purification level (in terms of number of contaminating bands) compared to rBulIFN-T8, we tried to examine its functional activity by antiviral assay. Interestingly, the antiviral assay on CHPV revealed that 0.067×10^{-7} mg of rBulIFN-T1a2 protein was able to inhibit CPE up to 50% exhibiting a specific antiviral activity of 1.5×10^8 antiviral units/mg of protein. Compared to our previous work [41] the antiviral activity in this study was more, which may be due to the different cell line and virus used.

5. Summary/conclusion

In buffalo at least 13 distinct *ifnt* gene variants exist which are transcribed into mRNA. These *ifnt* gene variants are supposed to be encoding eight different proteins during early pregnancy by the buffalo conceptus. IFN-T seems to be a multicopy gene with small variations. Based upon frequency of obtaining a particular type of sequence, IFN-T1 may be the major isoform expressed at mRNA level in the buffalo trophoblast cells. All BulIFN-T are closely related to bovine, caprine and ovine IFN-T. IFN-T in buffalo has 23 N-terminal amino acids as signal peptide. Majority of the isoforms seem to be N-glycosylated and heavily phosphorylated in 2/3rd C-terminal region. Relative abundance of individual isoforms can be

further validated for its functional significance. Out of 8 isoforms, IFN-T1a2 and IFN-T8 were successfully expressed in *E. coli* expression system and purified through IMAC chromatography. However, both yield and purification of protein may be enhanced further.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.pep.2016.02.005>.

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