Indian Journal of Biotechnology
Vol. 4, July 2005, pp. 363-366

Cloning, characterization and expression of bovine (*Bos indicus*) tumour necrosis factor-α

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Received 23 January 2004; revised 2 August 2004; accepted 16 August 2004

The gene for tumour necrosis factor-alpha (TNF-α) was amplified from cDNA pool prepared from LPS-stimulated bovine peripheral blood mononuclear cells isolated from Indian cattle. The amplified TNF-α gene was cloned and nucleotide sequences were determined. Homology comparison of nucleotide and predicted amino acid sequences revealed similarity at nucleotide and amino acid level in both exotic cattle and goat. The coding sequence of mature TNF-α (without its signal sequence and transmembrane anchor) was expressed as fusion protein with N-terminal polyhistidine using prokaryotic expression vector. The expressed protein was present as insoluble inclusion bodies. The recombinant protein was solubilized with urea and purified using Ni-agarose affinity chromatography. The purified recombinant TNF-α was characterized in SDS-PAGE and in western blotting.

Keywords: *Bos indicus*, cDNA sequence, expression, TNF-α, prokaryotic, recombinant
IPC code: Int. Cl.[7] C12N15/10, 15/28

Introduction

Tumour necrosis factor-alpha (TNF-α) is a soluble protein that causes damage to tumour cells but has no effect on normal cells[1]. It is an important inflammatory cytokine and has a central role in the networking to modulate the production and functional activity of several inflammatory cytokines[2]. Although its original activity was recognized against tumour cells, it is now known to play a significant role in immune and inflammatory responses as well as in the pathogenesis of many human and animal diseases and in cell proliferation and differentiation[3]. The nucleotide sequences for mouse, human, rabbit, pig, cat, sheep and exotic cattle TNF-α have been reported. Human, murine and feline TNF-α cDNA have been cloned and expressed in prokaryotic and eukaryotic expression systems[4-7]. The requirement of large quantities of TNF-α for immunotherapy and to produce antisera for diagnostic use has concerted the efforts towards production of TNF-α in large quantities using recombinant DNA technology. Various recombinant proteins, including cytokines have been expressed in prokaryotic expression system. These recombinant proteins have been utilized for therapeutic purpose and to raise monoclonal or polyclonal antibodies[8].

In this study, the authors report amplification of gene for TNF-α from Indian cattle (*Bos indicus*). The amplified gene was characterized by nucleotide sequencing and coding sequence of mature TNF-α gene was cloned into prokaryotic expression vector for production of recombinant bovine TNF-α.

Materials and Methods

Isolation of Total RNA and cDNA Synthesis

The bovine peripheral blood mononuclear cells (PBMCs) and were separated by density gradient centrifugation following the method described earlier[9] and stimulated with lipopolysaccharide (LPS) at the concentration of 10 µg per ml and incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. After 24 h, the stimulated cells were washed with PBS and total RNA was isolated using Trizol LS reagent (LifeTechnologies, New York) following the manufacturer's instructions. cDNA was synthesized from isolated total RNA using oligo (dT) primer (Promega, Madison, WI) following the method described earlier[10].

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Amplification of Full TNF-α Gene by PCR

The gene for bovine TNF-α was amplified from cDNA using TNF-α specific oligonucleotide primers (forward: 5'-GCC ACA AGG CTG TCC TGT CTC-3', reverse: 5'-ACA GGG CGA TGA TCC CAA AG-3') designed based on the sequence information reported earlier for goat TNF-α (GenBank Accession No. D86587). PCR reaction was performed using 2 µl of cDNA along with forward and reverse primers (50 pmol each), 200 µ*M* of dNTPs, 1.5 m*M* $MgCl_2$ and 3 U of Expand^{TM} High fidelity DNA polymerase (Roche, Mannheim, Germany). The amplification cycle was 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min and amplification at 72°C for 2 min. The PCR amplified product was analysed on 1% agarose gel along with DNA molecular weight marker.

Cloning and Characterization of Bovine full TNF-α Gene

The amplified full TNF-α gene fragment was gel purified and ligated into pGEM-T-Easy vector (Promega, Madison, WI)) following manufacturer's instructions. The recombinant plasmid (pGEM-fTNF-α) was characterized using *EcoRI* restriction digestion and analysed on 1% agarose gel for the insert. The TNF-α gene in recombinant plasmid pGEM-fTNF-α was sequenced using ABI PRISM 377 Version 3.0 DNA sequencer (Applied Biosystems, Foster City, CA) and sequences were analyzed using DNA Star software (Lasergene, Madison, WI).

Cloning of Mature form of TNF-α Gene into Prokaryotic Expression Vector

The mature form of TNF-α gene (without its signal sequence and transmembrane anchor) was amplified using pGEM-fTNF-α plasmid as target in PCR. The oligonucleotide primers used were Forward: 5'-GTT CAG GGA TCC AGG TCC TCT TCT CAA GCC TCA AG-3' with *BamHI* restriction site underlined and Reverse: 5'-CTG CAG AAT TCC AGG GCG ATG ATC CCA AAG-3' with *EcoRI* restriction site underlined. PCR reaction was performed using 0.5 µl of pGEM-TNF-α along with forward and reverse primers (50 pmol each), 200 µ*M* of dNTPs, 1.5 m*M* $MgCl_2$ and 3 U of ExpandTM High fidelity DNA polymerase (Roche, Mannheim, Germany). The amplification was 30 cycles of denaturantion at 94°C for 1 min, annealing at 55°C for 1.5 min and amplification at 72°C for 2 min. The PCR amplified product was gel purified and cloned into pRSET prokaryotic expression vector (Invitrogen, Carlsbad, CA). The recombinant plasmid pRSET-mTNF-α was characterized by double digestion with *BamH*I and *EcoR*I restriction endonucleases for presence of insert and its orientation.

Expression, Purification and Characterization of Mature TNF-α (m-TNF-α) in *E. coli*

The pRSET-mTNF-α plasmid with mTNF-α gene in correct orientation was transformed into *E. coli* BL21(DE3)pLyS expression host following manufacturer's instructions. The pRSET-mTNF-α plasmid carrying *E. coli* BL21(DE3)pLyS colony was grown at 37°C in LB broth containing 50 µg per ml ampicillin. When OD_{600} of the broth reached to 0.6, it was induced with 1 mM of isopropyl-β-D-galactopyranoside (IPTG) (Promega, Madison, WI) and cells were continued to grow further for 4-6 h. The induced *E. coli* cells were harvested by centrifuging the broth at 3,000 rpm for 10 min and freeze-thawed two times. The recombinant mTNF-α with 6 histidine residues at N-terminal end of the protein was purified under denatured condition purified using Ni-NTA slurry (Qaigen, Valencia, CA) following the instructions by the manufacturer. Briefly, the bacterial pellet was resuspended in lysis buffer (8 M urea, 0.1 M NaH_2PO_4, pH 8.0) and mixed with Ni-NTA slurry. The recombinant protein bound to Ni-NTA slurry was washed twice with wash buffer (8 *M* urea, 0.1 *M* NaH_2PO_4, *p*H 7.0) and eluted with elution buffer (8 *M* urea, 0.1 *M* NaH_2PO_4, *p*H 4.5). The eluted recombinant protein was separated on 12% SDS-PAGE and stained with coomassie brilliant blue. The eluted recombinant protein was characterized as recombinant mTNF-α in western blot using RGS monoclonal antibody specific to polyhistidine tag (Qiagen, Valencia, CA).

Results and Discussion

The total RNA isolated from LPS-stimulated bovine PBMCs showed the presence of bovine TNF-α mRNA in RT-PCR. A fragment of 752 bp using bovine TNF-α gene specific primers was obtained in RT-PCR amplification (Fig. 1). For sequence analysis, the amplified fragment was cloned into pGEMT-Easy vector. The full TNF-α (fTNF-α) gene fragment as insert could be released from recombinant plasmid pGEM-fTNF-α on digestion with restriction endonuclease *EcoR*I (Fig. 1). The insert fragment was released with *EcoR*I because this restriction site was

present on pGEMT vector multiple cloning sites flanking the fTNF-α gene insert. The nucleotide sequence of insert fTNF-α gene was determined and submitted to GenBank (Accession no. AY221122).

On sequence analysis of the amplified and cloned 752 bp DNA fragment, an ORF consisting of 705 bp, encoding 234 amino acid long TNF-α protein, was identified. The mature soluble form of TNF-α from exotic bovine was predicted to have 186 amino acids, deleting 84 amino acid signal peptide[1]. The ORF for TNF-α amplified in the present study is well corroborated with the sequence reported earlier for exotic bovine[10] and goat (GenBank Accession No. D86587). The alignment of the cDNA sequence of TNF-α from *Bos indicus* (in this study) revealed five nucleotide changes (99.1% similarity) in coding region of cDNA compared with *Bos taurus*[10] indicating close relationship with exotic cattle. However, there was no change at amino acid level as altered codons also coded for same amino acids. There were 28 nucleotide changes (95.9% similarity) compared with goat TNF-α coding sequence.

TNF-α is a type II transmembrane protein with predicted molecular weight of 25.5 kDa. There exists an extracellular mature soluble form (17.3 kDa) derived by proteolyic cleavage without its signal sequences and transmembrane anchor[1]. This soluble form of TNF-α is the only active protein executing variety of functions. In this study, the coding sequence for mature soluble form of TNF-α (mTNF-α), without its signal sequence and transmembrane anchor was amplified in PCR using primers designed to delete these regions. The amplified fragment of 491 bp was cloned into pRSET prokaryotic expression vector and analysed for presence of insert and its orientation in recombinant plasmid pRSET-mTNF-α using restriction endonucleases (Fig. 2).

For expression, the pRSET-mTNF-α recombinant plasmid was transformed into *E. coli* BL21(DE3)pLyS and induced with IPTG. After purification under denaturing conditions using Ni-agarose affinity chromatography, the purified protein was observed as single protein with a molecular weight of 21 kDa on SDS-PAGE (Fig. 3A). The recombinant protein with polyhistidine fusion tag facilitated the easy purification of fusion protein. Most of the expressed protein in our study was found as insoluble inclusion bodies. The formation of inclusion bodies of over produced protein and its renaturation are common with prokaryotic expression system[11]. The expressed recombinant protein, in our study, was eluted with elution buffer of *p*H 4.5 and not with that of *p*H 5.9 indicated the strong binding with Nickel in affinity chromatography. The observed molecular weight of recombinant mTNF-α in the present study was 21 kDa as opposed to expected molecular weight of 17 kDa predicted using nucleotide sequence. This increase in size of

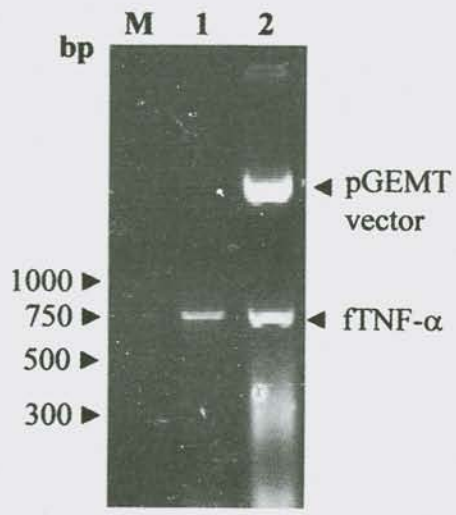

Fig. 1—RT-PCR amplification and cloning of full TNF-α (fTNF-α) gene. Lane 1: RT-PCR amplified fTNF-α gene, Lane 2: pGEM-fTNF-α recombinant plasmid digested with *EcoRI*, M: PCR marker

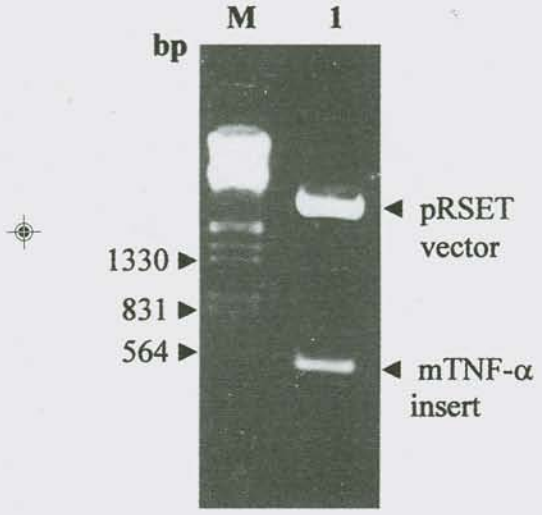

Fig. 2–Cloning of mature TNF-α (mTNF-α) coding sequence into pRSET prokaryotic expression vector: Lane 1, pRSET-mTNF-α plasmid double digested with *BamHI* and *EcoRI*, M: λ DNA digested with *HindIII* and *EcoRI* marker

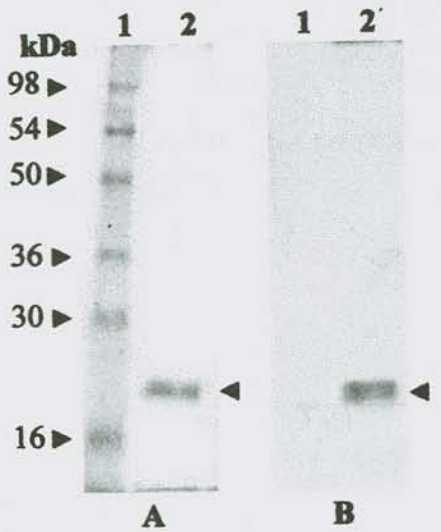

Fig. 3–SDS-PAGE (A) and western blot (B) analysis of purified recombinant mTNF-α protein, Lane 1, Protein molecular weight marker & Lane 2, purified recombinant mTNF-α protein

recombinant protein was due to N-terminal tag and other in frame amino acids similar to the observation reported earlier for other proteins[8,12]. This purified protein reacted with mouse RGS monoclonal antibody specific to polyhistidine tag in western blot analysis (Fig. 3B).

This recombinant bovine mTNF-α protein expressed in *E. coli* can be purified in bulk as antigen for preparation of monoclonal antibody against bovine mTNF-α or can be refolded to prepare a biologically active molecule to be used for therapeutic purpose.

Acknowledgement

Authors are thankful to the Director, Indian Veterinary Research Institute, Izatnagar for providing necessary facilities to carry out the work. The help extended by incharge National Project on Rinderpest Eradication (NPRE), quality control (QC) is highly acknowledged.

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