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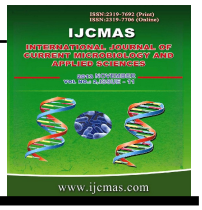

Original Research Article

Expression, purification and characterization of recombinant Heat Shock Protein 70 (HSP70) from sheep and goat species

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K e y w o r d s

Sheep; Goat; Heat Shock Protein 70; cloning; expression; confirmation.

A B S T R A C T

Molecular chaperones, including the heat-shock proteins (HSPs), are a ubiquitous feature of cells in which these proteins cope with stress-induced denaturation of other proteins. In this study 1926 bp fragment of Heat Shock Protein 70 (*orf*) was amplified, cloned, sequenced and characterized in sheep (*Ovis aries*) and goat (*Capra hircus*). DNA was isolated from lymphocytes, used for PCR amplification of Hsp70 gene, cloned in pGEM-T easy vector and sequenced. Sequence analysis revealed 1926-bp long open reading frame of Hsp70 gene encoding 641 amino acids in these species. The predicted amino acid sequence of *Capra hircus* showed 98% identity with *Ovis aries*, *Bos indicus*, *Bubalus bubalis* and more than 90% indentity with *Canis familiaris*, *Sus scrofa* and *Homo sapiens* Hsp70 protein sequences. On phylogenetic analysis, sequences in study clustered away from the avian sequences, closest among bovid group and farthest from primate group. The expression of this gene was in prokaryotic expression vector pPRoExHTa producing a recombinant protein of ~70kDa. His tagged protein purified by Ni-NTA affinity chromatography under denaturing conditions which was confirmed by western blotting using Ni-NTA HRP conjugate and 4 chloro-1-napthol as substrate. Recombinant Hsp70 was produced in large quantity using mentioned protocol in a cost effective manner.

Introduction

Increased expression of stress proteins is one of the most conserved stress response mechanisms. Elevated synthesis of a few proteins following exposure to heat or other stresses occurs in all organisms studied, ranging from prokaryotic bacteria to mammals (Latchman, 1999). It is now well established that many stress proteins are encoded by multi-gene families encoding proteins with similar but distinct features. Molecular chaperones belong to several highly conserved ubiquitously distributed families of proteins (Macario *et al*., 2004), most of which are ATPases, the general role of which is to control protein quality and regulate protein structures in cells. All chaperones share the ability to scrutinize various protein structures in the crowded cellular environment (Ellis, 2006). They distinguish between unfolded,

misfolded and native protein conformers. High-affinity chaperone binding to exposed hydrophobic segments of unfolded 'client' proteins is endowed with the passive function of preventing the formation of stable, irreversible protein aggregates, thereby facilitating spontaneous native-protein folding (Hartl, and Hayer-Hartl, 2002). Early forms of mutation- or stress-induced protein aggregates not only lack native activity, but might cause direct damage to membranes (Rochet *et al*., 2004).

Protein aggregates can also seed and propagate (Diamant *et al*., 2000) misfolding and aggregation to other labile or aggregation-prone proteins in the cell (Ben-Zvi, and Goloubinoff, 2002; Gidalevitz, *et al*. 2006), as in the case of prions (Serio, and Lindquist, 2001). Because of the cytotoxicity of protein aggregates, molecular chaperones are central components of the cellular machinery against protein-misfolding diseases and aging (Hinauld *et al*., 2006). The best-known stress proteins that are induced in stressed cells are members of the heat shock protein (HSP) family. Hsp70 is the major inducible member of the heat shock protein family.

The levels of Hsp70 in cells have been correlated with tolerance to a wide variety of stresses which include environmental insults such as heat shock, heavy metals (Wagner *et al*., 1999), osmotic stress (Kurz *et al.*, 1998), as well as physiologic stresses such as ischemia (Nowak *et al*., 1990; Kumar and Tatu, 2000), oxidative stress, etc. Although there is overwhelming evidence for the cytoprotective role of Hsp70, most of the studies have been performed in model systems such as isolated human cells (Gutsmann *et al*., 1998) and to a lesser extent in animal models of stress.

We have initiated a program to study heat shock protein in the background of heat stress condition experienced by farm animals. In addition to the usefulness of heat shock proteins as markers to gauge stress tolerance, the study may provide new approach to counter stress tolerance in farm animals. In this study we describe cloning, sequencing, induction of prokaryotic expression, purification and confirmation of recombinant Hsp70 in sheep and goats. It is concluded that the relevance of heat shock proteins as stress markers and their potential in improving stress tolerance in animals.

Materials and Methods

Bacterial strains and plasmids

Prokaryotic expression vector pProExHTa (Invitrogen, USA) was used as vector for the expression experiment. pGEM-T Easy^{TM} (Promega, USA) TA cloning vector for the cloning experiment. *E. coli* strain DH5α was used as host for preparation of competent cells, expression experiment and for its culture, Luria-Bertani broth (1.0% tryptone, 0.5% yeast extract, and 1% NaCl) and Luria-Bertani agar (1.0% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar) medium were used. X-gal (40 mg/ml), IPTG (100 mg/ml) and Ampicillin (100 mg/ml) for the preparation of agar plates for growth, incubation was done at 37^0C and ampicillin was added to the medium for selection, wherever necessary. All media were procured and prepared as per the manufacturer's recommendations (HiMedia, Mumbai).

Blood collection and DNA isolation

Whole blood was collected in the

heparinized collection tubes from healthy sheep and goats which were reared under normal feeding practices. The buffy coat was collected by centrifugation and DNA was extracted from the buffy coat containing leukocytes by standard phenol/chloroform method (Sambrook *et al*., 1989). Concentration and purity of DNA was assessed by UV spectro-photometry using Nanodrop system (Nanodrop 2000C, Thermo Scientific, USA).

PCR amplification of HSP70 orf and cloning

In order to amplify the HSP70 *orf*, primers were designed after thorough analysis of the available sequences of the HSP70 gene on NCBI database by clustalW. Primers were custom synthesized (Sigma-Aldrich, USA). Restriction endonuclease sites for *NcoI* and *XhoI* enzymes were included in the forward and reverse primers respectively, (underlined and italicized), for directional cloning of the PCR product. Three additional base pairs (cgc) were added to the extreme 5' end of the forward and reverse primers for providing proper binding of the restriction enzymes at their recognition sequences. Two bases pairs (CC; shown in bold) were included after restriction enzyme recognition site (i.e. before start codon ATG) of the forward primer to maintain the reading frame of the expressed recombinant protein.

The sequence of the primer pair used is HSP70 F: 5' CGC *CCATGG* CC ATGGCGAAAAACATGGCTATCGGC 3'and HSP70 R: 5' CGC *CTCGAG* CTAATCCACCTCCTCAATGGTGGGG C 3'. The PCR assay was optimized using 25µl reaction mixture to get a single specific amplicon of desired size. Final standardized PCR reaction contained 1X PCR buffer, 2.5mM $MgCl_2$, 200 µM dNTP mix, 20pM each of forward and reverse primers, 2.5U of *Taq* DNA polymerase and approx. 150 ng of template DNA. Cycling conditions standardized for PCR amplification included one cycle of initial denaturation at 94˚C for 5 min followed by 35 cycles each of denaturation (94˚C for 1 min), annealing (50˚C for 1 min) and extension (72˚C for 1.5 min) followed by final extension at 72˚C for 10 min. The PCR product was visualized after electrophoresis at 80 V for approx. 2 hour. Electrophoresis was carried out on 1.5% agarose gel prepared in 0.5X TBE buffer and containing 0.5µg/ml ethidium bromide and photographed on ChemiDoc XRS gel documentation system (Biorad, USA).

Gel purified PCR product was ligated with linearized pGEM-T Easy cloning vector (Promega, USA); for this reaction was set up on ice and after mixing, ligation mixture was incubated at 4˚C for overnight. Competent *E.coli* (DH5α) cells prepared by calcium chloride ($CaCl_2$) method and transformation of ligated plasmid into DH5α competent cells was done. Heat shock was given at 43^0C for 90 sec followed by immediate cooling of the tubes on ice for 5-10 min. The cells were plated on to LB agar plates containing ampicillin, X- gal and IPTG and incubated at 37^0C for 15-18 hr. White Recombinant colonies were picked and inoculated in LB containing ampicillin. Plasmid was isolated by alkaline lysis method (Sambrook and Russell, 2001) then restriction double digestion of the isolated plasmids was carried out with *NcoI* and *XhoI* enzymes at 37˚C for overnight and resulted insert analyzed on 1.5% agarose gel. One clone was selected and plasmid isolated from this clone by Qiaquick plasmid isolation kit (Qiagen, Germany). The isolated plasmid confirmed by restriction digestion.

Sequence Analysis

After the confirmation one clone is selected and subcultured in LB broth at 37^0 C for overnight. Subcultured clone was sent for sequencing "DNA sequencing facility" at Department of Biochemistry, Delhi University, New Delhi (India). The sequence obtained was compared to the gene sequence for the HSP70 protein of sheep, goat, buffalo and cattle available at GenBank *(www.ncbi.nlm.nih.gov)* and submitted to NCBI genebank and accession number was obtained. Sequence alignment was performed using Clustal W method of MegAlign module in DNAstar Version 4.1 Inc., USA. Sheep and goat protein structure was predicted by SWISS-MODEL Workspace *(http://swissmodel. expasy.org/workspace/)* using PDB viewer software. Phylogenetic tree based on the evolutionary distances was constructed from nucleotide sequences using MEGA 4 software. Based on the nucleic acid alignment, the number of synonymous substitution per synonymous site (dS) and number of nonsynonymous substitution per nonsynonymous sites (dN) were estimated, and neutral (dS = dN), positive (dN > dS), or purifying (dN < dS) selections were tested with a codon-based *Z* test using Nei Gojobori method.

Construction of recombinant expression plasmid

After standardization, PCR for Hsp70 genes of sheep and goat were put in bulk (100µl) under similar conditions. PCR product was run on 0.8% agarose gel, prepared in 0.5X TBE buffer for gel purification. Gel purification of the Hsp70 gene product was done using QIAquick gel extraction kit as per the protocol provided by manufacturer (Qiagen, USA). Eluted PCR product was reconfirmed for purity by electrophoresis on 1.5% agarose gel. Concentration and purity of DNA was assessed by UV spectrophotometry using Nanodrop system (Nanodrop 2000C, Thermo Scientific, USA). Gel purified Hsp70 PCR products and pProExHTa prokaryotic expression vector (Invitrogen, USA) were double digested with *NcoI* and *XhoI* restriction enzymes at 37^0C for overnight. Double digested products were again purified by QIAquick gel extraction kit (Qiagen, USA) after running on 0.8% agarose gel.

Double digested and gel purified pProExHTa and Hsp70 PCR products were then ligated in 20µl reaction set up (containing 5.5µl vector, 11µl PCR product, 1.5µl T4 DNA ligase and 2µl T4 DNA ligase buffer) in a microcentrifuge tube and left for overnight at 14˚C. *E. coli* (DH5α) was grown in SOB medium and competent cells were freshly prepared by calcium chloride ($CaCl_2$) teatment. Transformation of ligated plasmid into freshly prepared competent cells was done by heat shock at 42^0C for 1.5 min followed by immediate cooling of the tubes on ice for 10 min. 0.8 ml of the SOC medium was added to the tube and incubated at 37^0C for 90 min. The cells were plated on LB agar plates containing ampicillin (100µg/ml) and incubated at 37^0C for 15-18 h. Six colonies were randomly picked from plates and inoculated in LB broth containing ampicillin (100µg/ml). Plasmids were isolated by alkaline lysis method following standard plasmid extraction protocol (Sambrook and Russell, 2001). Clones were confirmed for presence of insert by restriction double digestion of the isolated plasmids (with *NcoI* and *XhoI*) and also by PCR using isolated plasmids as template. Results were analyzed on 1.5% agarose gel prepared in 0.5X TBE buffer and

photographed on ChemiDoc XRS gel documentation system (Biorad, USA).

Expression of recombinant Hsp70:

One positive clone of each that released specific size inserts and gave specific amplicons in PCR was selected (Hsp70GT/2 for goat and Hsp70SH/4 for sheep). These clones were further used for induction of expression of recombinant Hsp70. For induction of expression, the selected clones were grown in LB (containing ampicillin 100µg/ml) to the level of 0.6 OD (at 600nm). Induction was done by adding IPTG to a final concentration of 0.6 mM. After induction by IPTG, 1.5 ml sample was collected at hourly intervals up to 6 h, pelleted and stored at -20^{0}C until analyzed by SDS-PAGE. Un-induced sample was also collected before addition of IPTG as negative control and processed as above. For SDS-PAGE analysis, 100µl of 2X sample buffer was added to the pellet and boiled for 10 min. In SDS-PAGE, 50 µl of the samples were loaded along with the PageRuler^{TM} Prestained protein ladder (Fermentas, USA) in one lane. The results were photographed on ChemiDoc XRS gel documentation system (Biorad, USA).

Purification of recombinant Hsp70 proteins:

Expressed recombinant His-tagged Hsp70 fusion proteins were purified by Ni-NTA affinity chromatography using protocol provided by the manufacturer (Qiagen, USA). Selected clones were grown in 50 ml LB broth and induced by IPTG as described above. After induction, samples were collected and pelleted. The collected pellets were re-suspended in 10 ml of buffer B (100 mM NaH_2PO_4, 10 mM Tris-Cl and 8M Urea, pH-8.0). Cells were lysed by sonication at an amplitude of 15.0 for 10 cycles (30 seconds pulse-on followed by 30 seconds pulse-off) using microtip (Misonix, USA). Sonicated cell lysates were centrifuged at high speed (13000 rpm for 30 min at room temperature) to remove cell debris. The supernatant was collected and mixed with 2.0 ml of Ni-NTA agarose (Qiagen, USA) and left on gyroshaker for 2 h for efficient protein binding with Nickel. The mixture was passed through a column containing porous silica and three times washed with buffer C (100 mM NaH_2PO_4, 10 mM Tris-Cl and 8M Urea, pH-6.3). Finally the proteins were eluted in small fractions using buffer E (100 mM NaH_2PO_4, 10 mM Tris-Cl and 8M Urea, pH-4.5). Collected protein fractions were run on SDS-PAGE to detect presence of purified protein. The results were photographed on ChemiDoc XRS gel documentation system (Biorad, USA). Small sized contaminant proteins (if any) and salts (urea) were removed from purified protein by dialysis against 1X PBS using a dialysis tubing of 12kDa cut-off value.

Confirmation of recombinant Hsp70:

Western blotting was performed for confirmation of purified and dialysed His-tagged Hsp70 fusion proteins by using nitrocellulose membrane (Millipore, USA), Ni-NTA HRP conjugate (Qiagen, USA) and 4-chloro-1-napthol (Sigma, USA) as substrate following standard protocol (Qiagen, USA). For western blotting, following separation on SDS-PAGE, protein was electro blotted on to NCM using a semidry blotter (Atto, Japan) at 20mA for 2 h. Blocking was done for 1 h with 3% BSA (SRL, India) prepared in TBS buffer. Washing was done three times with TBS-tween 20 (TBS-T) buffer each time for 10 min. Ni-NTA HRP conjugate

(Qiagen, USA) was diluted to 1:1000 in TBS-tween 20 (TBS-T) buffer and NCM was soaked into it for 1 h. Again, washing was repeated thrice with TBS-T buffer for 10 min each. Membrane was then soaked in staining solution (18 mg of 4-chloro-1-napthol in 6 ml of methanol mixed to 24 ml of 1X Tris-saline and finally 60 µl of 30% H_2O_2 added). Development of color was observed and reaction was stopped by rinsing the membrane in distilled water. The results were photographed on ChemiDoc XRS gel documentation system (Biorad, USA).

Results and Discussion

PCR amplification and cloning:

The concentration of DNA of goat and sheep was checked by analyzing OD 260/280 ratio which was found in the range of 1.6-1.8 indicated the purity of the DNA, and the yield was obtained in range of 240–290 ng/µL. PCR amplification was carried out using extracted DNA. Agarose gel electrophoresis revealed 1926-bp PCR product of Hsp70 gene on 1.5% agarose gel (Figure 1). The gene insert (1926 bp) was released from the recombinant pGEM-T easy plasmid using *EcoRI*. GAATTC is restriction site for *EcoRI* which releases gene insert from recombinant plasmids.

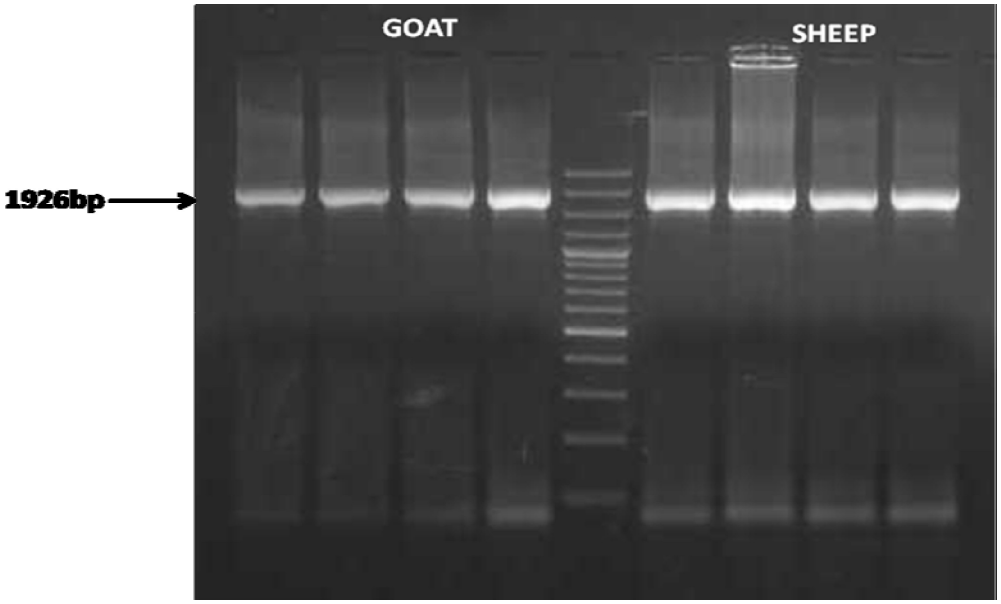
The plasmids were further characterized by *NcoI* and *XhoI*. Restriction sites were incorporated in forward and reverse primers respectively for further expression of gene. The sequencing of this plasmid revealed that Hsp70 of goat has full length *orf* of 1926 bp. The sequence was submitted to NCBI Genbank, and the accession number: JN604433, JN604434 for sheep and goat Hsp70 gene complete CDS was obtained.

Sequence analysis:

The nucleotide sequence and predicted amino acid sequence were aligned and compared with all Hsp70 *orf* sequences of different domestic species namely, sheep (JN604433), buffalo (JF502845), cattle (JN604432), pig (AK 235308), dog (AB114672) and human (AK 301243) using ClustalW method of MegAlign module of DNAstar Version 4.1 Inc. USA, which revealed the nucleotide substitutions. The entire nucleotide sequence of sheep Hsp70 gene shows 98% homology with cattle, buffalo, goat and yak, 96% with camel, 95 % with pig, 94% with dog and 93% with human which indicates close evolutionary relationship. Among the twenty one nucleotides substitutions in DNA sequence of Hsp70 gene of goat, as compared to sheep, changes at amino acid positions 9, 100, 121, 184, 263 and 569 resulted in substitutions (Figure 2). Inferred amino acid sequence of 641 residues of goat Hsp70 was 98.9% similar to sheep, 98.8% to cattle, 98.9% to buffalo, 95.7% to pig, 94.8% to dog and 95.7% to human sequence (Figure 3). The results indicate that Hsp70 nucleotide and deduced amino acid sequence is highly conserved across the species.

Similar results was shown by Gade *et al*., (2010) amino acid sequence of goat Hsp70 gene was 98.6% similar to cattle, 95.9% to buffalo, 98.4% to yak, 100% to sheep (partial), 98% to pig, 98.1% to horse, and 97.7% to human sequence. Pelham (1982) reported that Hsp proteins are highly conserved both in protein coding sequence and in regulatory sequence. Gutierrez and Guerriero (1995) found that amino acid sequences 10–16 and 131–139 were highly conserved in the Hsp70 family of proteins. In buffalo Hsp70, amino acid isoleucine

Figure.1 PCR amplification of complete HSP70 gene from Cattle



M: GeneRulerTM 1 Kb plus DNA ladder (Fermentas, USA)

replaced valine at position 9, aspartic acid is replaced by histidine at position 186 and isolucine is replaced by serine at position 297. Sequence from 10–170, 212-262 and 298-568 was found to be conserved (Figure 3). Morimoto *et al.* (1986) characterized chicken Hsp70 gene and found that chicken Hsp70 cDNA sequence and deduced amino acid sequence is 80% identical to human, Hsp70 cDNA sequence and deduced amino acid sequence, whereas 73% identical to Drosophila Hsp70 cDNA sequence and 71% identical to Drosophila Hsp70 amino acid sequence. The amino acid sequence of Hsp70 gene in canine showed 90%–95% sequence similarity with bovine, human, and mouse Hsp70 proteins (Kano *et al*., 2004)

Phylogenetic analysis:

Based on the nucleic acid sequences of Hsp70 full length *orf*, phylogenetic tree was drawn by Mega 4.1 (Tamura *et al*., 2007) considering 1,000 bootstrap values. It was found that ruminants and monogastrics are derived from different clusters according to their closer evolutionary relationship. Among ruminants, goat, sheep, cattle and buffalo, might have evolved from a common ancestor; pig positioned in between and diverged early from the bovid ancestors. Selection pressure, as determined by codon-based *Z* test using the Nei Gojobori method, revealed that at 5% level of significance. Thus, Hsp70 might have evolved by positive selection among these species. Goat and sheep Hsp70 gene showed identical lineage. However, cattle, buffalo, yak, camel, pig and human are having similarity with goat having different lineage. Chicken, quail, guinea fowl and goose sequences show dissimilarities suggesting different ancestry (Figure 4).

Figure.2 Amino acid sequence alignment of HSP70 of goat with sheep, cattle, buffalo, dog, pig and human

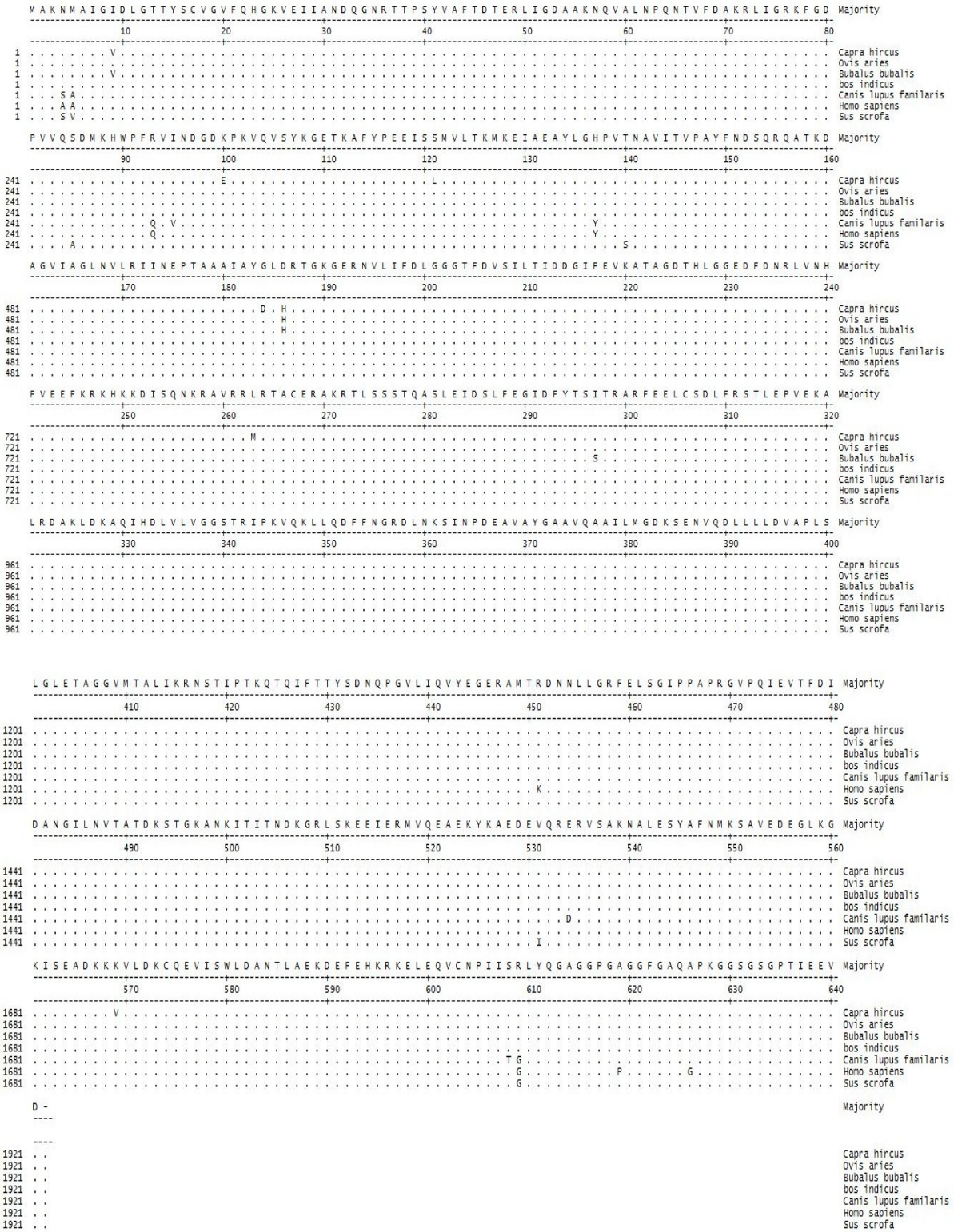


Figure.3 Percent identity of goat and sheep predicted protein sequences with other species

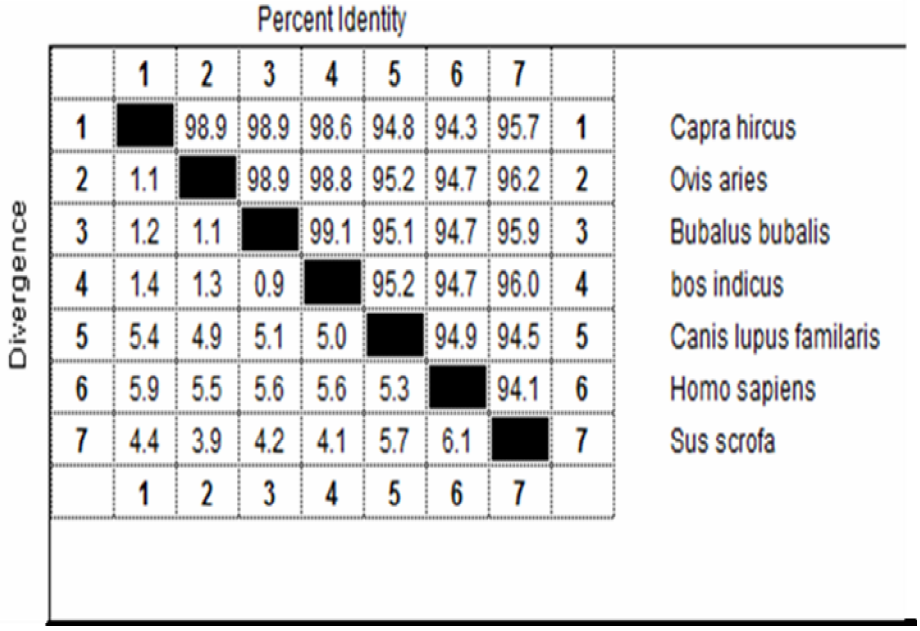


Figure.4 Phylogenetic tree showing relationship between goat and sheep with other species of HSP70 gene

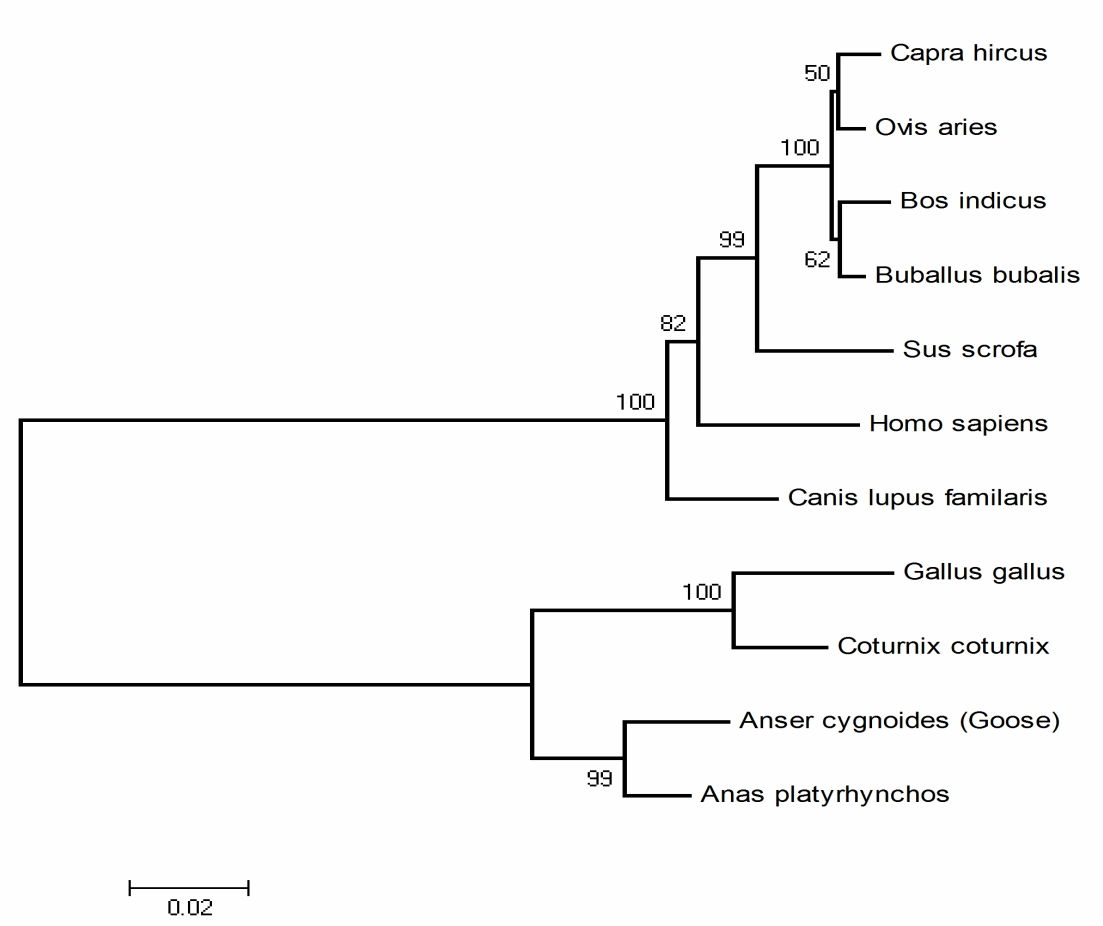
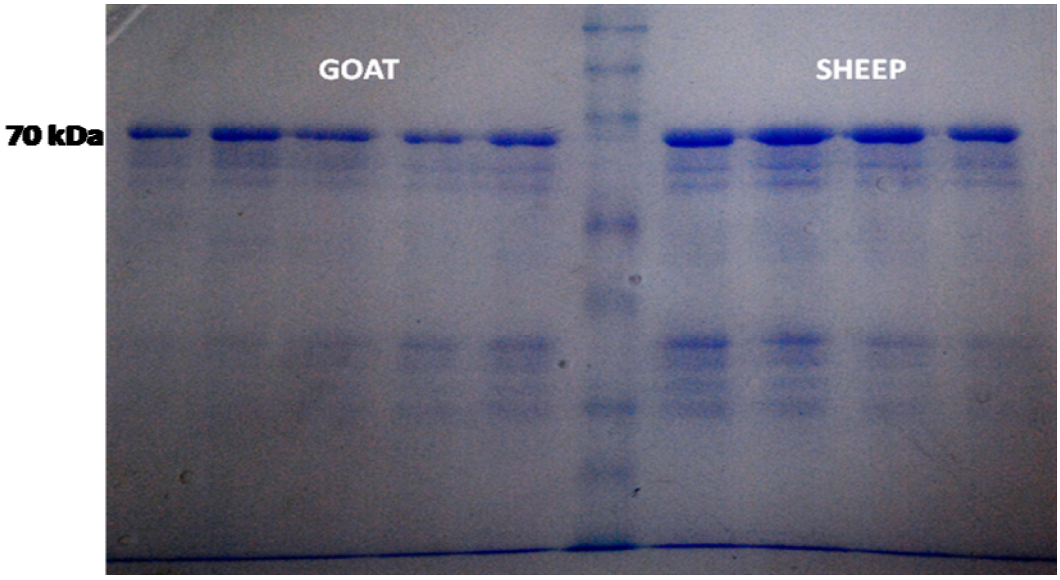
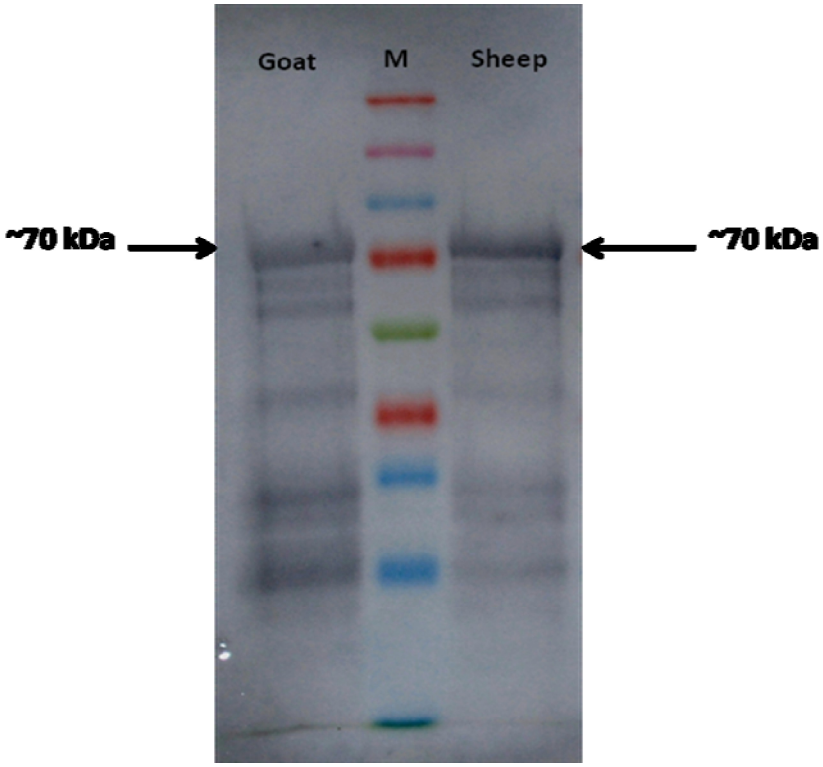


Figure.5 His tagged purified protein by Ni NTA affinity chromatography
Purified protein showing dark band at ~70kDa



M: PageRulerTM Pre-stained protein ladder (Fermentas, USA)

Figure.6 Western Blotting for the confirmation of His-tagged *HSP70* protein



P: Recombinant HSP70 protein showing dark band at ~70kDa; M: SpectraTM Multicolor Broad range protein ladder (Fermentas, USA)

Comparison of predicted sheep and goat Hsp70 protein structure:

Predicted Hsp70 protein of goat possesses molecular weight of 70353.67 Da with 641 amino acids of which 81 Strongly Basic (+) Amino Acids (K,R) and 93 Strongly Acidic (-) Amino Acids (D,E). Further 222 hydrophobic amino acids (A,I,L,F,W,V) and 150 amino acids (N,C,Q,S,T,Y) polar in nature. Isoelectric point of Hsp70 protein of goat is 5.515 and has got charge of -10.657 at PH 7.0. Whereas sheep possesses 70293.63 Da with 641 amino acids of which 83 strongly basic (+) amino acids (K,R) and 91 strongly acidic(-) amino acids (D,E). Further 221 hydrophobic amino acids (A,I,L,F,W,V) 151 polar amino acids (N,C,Q,S,T,Y). Isoelectric point of Hsp70 protein of sheep is 5.924 and has got charge of -6.662 charge at PH 7. Molecular structure of Hsp-70 contains 44-kDa fragment (amino acid residues 1–386) at N-terminus contains 4 domains forming 2 lobes with a deep cleft between. 18-kDa fragment (amino acid residues 384–543) contains two 4-stranded antiparallel b-sheets and single a-helix. (Kiang J.G. and Tsokos G.C., 1998) 10-kDa fragment (amino acid residues 542–640 for Hsp-70) at C-terminus conserves EEVD terminal sequence. The N-terminal 44-kDa domain is ATPase domain; 18-kDa domain is peptide-binding domain; C-terminal 10-kDa fragment carries highly conserved EEVD terminal sequence, which is present in all eukaryotic Hsp-70 and Hsp-90 (Hightower, L. E *et al.*, 1994).

Prokaryotic expression and western blotting

Amplified Hsp70 gene product and pProExHTa prokaryotic expression vector was digested with *NcoI* and *XhoI*. Ligation reaction was set up and ligated product was successfully transformed in to freshly prepared *E. coli* DH5α competent cells as evident by appearance of numerous white/opaque colonies on LB agar plates. Transformation efficiency was found to be optimum. Randomly six colonies were picked and grown in LB and plasmids were isolated from them. The recombinant plasmids with Hsp70 gene were confirmed by restriction double digestion and PCR which resulted in release of a fragment of desired size 1926bp and amplification of desired size product as analyzed on agarose gel. PCR amplification using plasmids isolated from the clones also resulted in the specific size amplicon. For expression studies, one clone of each was selected and induced by IPTG which after SDS-PAGE (Laemmli,1970) analysis resulted in a thick band corresponding to ~70 kDa as compared to un-induced control as evident under coommasie brilliant blue R250 staining. Expression kinetics indicated that the maximum expression was at ~6h post induction.

Protein purification by Ni-NTA affinity chromatography resulted in a specific ~70 kDa band as detected by SDS-PAGE after CBB R-250 staining with few contaminant proteins (Figure 5). Report shows that the amount of a contaminating recombinant protein was approx. 75 kDa. When the recombinant Hsp70 was compared with the bovine skeletal-muscle Hsp70, they was observed that it migrated with the inducible Hsp70. These results demonstrated that the cloned protein with a mobility on that SDS PAGE identical with the recombinant protein (Gutierrez and Guerriero, 1995). Salt (urea) and small sized contaminant proteins (if any) were successfully removed from purified protein by dialysis against 1X PBS. Western blotting of purified and dialyzed Hsp70 fusion protein by using

nitrocellulose membrane (NCM), Ni-HRP conjugate and 4-chloro-1-napthol as substrate resulted in dark violet colored band development at a location corresponding to ~70 kDa in the pre-stained protein ladder (Figure 6).

The present study was undertaken to clone, sequence, express and purify recombinant Hsp70. Sheep and goat heat shock protein 70 mRNA encoding Hsp70 protein of 641 amino acid residues was found highly conserved among domestic animals. The study shows that prokaryotic expression vector pPROExHTa (Invitrogen, USA) can be successfully used for production of recombinant HSP70 protein in bulk and His-tagged protein can be purified to almost homogeneity for various downstream applications including production of hyperimmune antisera, monoclonal antibodies, development of rAgELISA for stress detection in animals or as vaccine adjuvant. Attempts are underway to evaluate its immunogenicity in experimental animal models and to develop monoclonal antibodies against Hsp70. Also to compare native and recombinant protein on 2D electrophoresis along with the protein structural characterization and confirmation of Hsp70 need to study.

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