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Short communication

Characterization and expression profile of complete functional domain of granulysin/NK-lysin homologue (buffalo-lysin) gene of water buffalo (*Bubalus bubalis*)

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ABSTRACT

Granulysin (GNLY)/NK-lysin (NKL) is an effector antimicrobial cationic peptide expressed in the cytotoxic and natural killer lymphocytes. We report here cDNA sequence (405 bp) encoding the complete functional domain of buffalo-lysin (bu-lysin), and its expression profile in the various tissues. The nucleotide sequence of bu-lysin exhibited >85% identity with the bovine lysin. Comparison of the deduced amino acid sequence of bu-lysin with those of GNLY/NKL of different species revealed the conservation of six cysteine (Cys) residues and five alpha helices. Unlike the homologues in other species, bu-lysin composed of 11 positively charged Lys residues as in equine. The expression of bu-lysin mRNA in the *in vitro* cultured lymphocytes was inducible and increased markedly ($p < 0.05$) in a dose dependant manner when incubated with Concanavalin A (ConA). The expression of bu-lysin mRNA in the different tissues was variable: comparatively higher in the spleen and lymph node, moderate in the uterine endometrium and low in the liver and kidney. These results indicate the existence and active expression of GNLY/NKL homologue in water buffalo having a significant influence in immune response.

1. Introduction

Antibacterial peptides play an important role in host defense against microbial pathogens (Boman, 1995). The natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), together with neutrophils and macrophages, are the primary effector cells of the immune system. These cells, on stimulation, release cytolytic granules, which contain a wide range of proteins, including those of perforin and the granzyme family, capable of causing membrane lysis and eventual cell apoptosis (Gamen et al., 1998). Jongstra et al. (1987) identified a novel gene from human T-cell lymphocyte, later renamed as granulysin (GNLY) due to its location in cytolytic granules of NK and CTL cells (Krensky, 2000). The gene encodes a peptide having broad lytic abilities against bacteria, fungi, protozoa, and parasites (Ernst et al., 2000; Gansert et al., 2003; Stenger et al., 1998). Subsequently, porcine homologue of human GNLY, referred to as NK-lysin (NKL), having similar structure and antimicrobial properties (Andreu et al., 1999; Jacobs et al., 2003) was identified from the intestinal tissue (Andersson et al., 1995a, 1996). The native as well as several synthetic GNLY/NKL peptide fragments displayed potent antimicrobial activity against several gram-negative and gram-positive bacteria (Andersson et al., 1995b; Andreu et al., 1999).

Antimicrobial peptides, although diverse in size, sequence, structure and spectrum, play an important role in immune responses of the host (Hancock and Diamond, 2000). Peptide mediated innate defense is an evolutionary

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old mechanism to combat microbial pathogen; therefore, it is quite likely that different species should evolve similar molecules to combat infection. During last decades several antimicrobial peptides have been isolated from a wide range of animal species. Further, the threat of the emergence of antibiotic-resistant bacteria and the non-availability of newer classes of antibiotics has prompted the need to identify novel antimicrobial peptides for the development of alternative therapeutics for both human and animal. Since the discovery of human GNLY (Jongstra et al., 1987), its homologues genes encoding GNLY/NKL peptides have been identified in different species including bovine (Endsley et al., 2004), equine (Davis et al., 2005), porcine (Andersson et al., 1995a), chicken (Hong et al., 2006) and catfish (Wang et al., 2006).

Water Buffalo (*Bubalus bubalis*) being a major contributor of milk production serves as one of the most important livestock species in India as well as in Southeast Asian countries (FAOSTAT, 2003). Further, the buffalo is a sturdy animal, well adopted to the tropics, and often anecdotally referred to as being resistant to many infectious diseases. However, only a few antimicrobial peptides of bubaline origin including β-defensins (Bera et al., 2007; Das et al., 2005), cathelicidin (Das et al., 2006), and cathelicidin-7 like antibiotic peptide (Das et al., 2008) have been characterized. Consequently, the immune mechanism behind the widely believed disease resistance is not known due to limited studies about bubaline immune effector molecules (Mingala et al., 2007). In this study, we report the cloning, characterization and the expression profile of the cDNA sequences encoding the entire mature peptide of GNLY/NKL homologue in buffalo, the bubaline-lysin (bu-Lys).

2. Materials and methods

2.1. Isolation of peripheral blood mononuclear cells (PBMC)

Venous blood was collected aseptically from healthy adult buffaloes. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation using Histopaque (Sigma, USA) following manufacturer's instructions. Briefly, 8 ml of PBS-diluted (1:1) blood samples were layered over 4 ml of Histopaque in 15 ml tube and centrifuged at $400 \times g$ for 30 min. PBMC at interphase were collected and washed three times with sterile PBS.

2.2. cDNA synthesis

The total RNA was isolated from PBMC using RNAgents system (Promega, USA) according to the manufacturer's instructions. The isolated RNA sample was treated with DNase using DNA-free™ DNase Treatment & Removal Reagents (Ambion, USA). The concentrations and purities of RNA preparations were determined spectrophotometrically at OD_{260} and OD_{280}. The total RNA was reverse-transcribed using Reverse Transcription (RT) System (Promega, USA) following the manufacturer's instructions. Briefly the cDNA was synthesized from approximately 2 μg total RNA using oligo-dT primers and avian myloblastosis virus reverse transcriptase in a final volume of 20 μl. The resultant first strand of cDNA was stored at −20 °C until use.

2.3. PCR amplification

A part of bu-lysin gene corresponding to the mature peptide was amplified using a pair of bovine-lysin primers (Forward: 5′CTGCTGCTCCAAGGAGAAGA3′ and Reverse: 5′GCAGTGGAGGGAGTTTGGT3′) as described by Endsley et al. (2004). PCR amplification was carried out in a total volume of 25 μl that contained 5 pmol of each primer, 5 μl of cDNA, 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM dNTPs and one unit of *Taq* DNA polymerase (Fermentas, USA). Amplification was carried out in a Thermal Cycler (Eppendorf, Germany) for 35 cycles in the following conditions: 94 °C for 2 min and 35 cycles of 30 s at 94 °C, 60 s at 60 °C, and 30 s at 72 °C and a final extension of 10 min at 72 °C. The amplification products were resolved by agarose gel (1%) electrophoresis and visualized using ethidium bromide (EtBr) staining under UV light.

2.4. Cloning and sequencing

The amplicon was purified using Gel Cleanup kit (Eppendorf, Germany) and cloned into pDrive vector (Qiagen, USA) following the manufacturer's instructions. Positive recombinant clones were identified using blue and white screening. Further, the presence of the insert was confirmed by restriction digestion and plasmid PCR. The positive clones were sequenced using an ABI PRISM automatic sequencer (version 2.0) using standard cycle conditions by Sanger's dideoxy chain termination method with the standard T7 and SP6 sequencing primers. The sequences were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST). The nucleotides as well as the deduced amino acid sequences were aligned with those of available species in the GenBank database using the Clustal method of MegAlign Programme of Lasergene Software (DNASTAR, USA). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007). Phylogenetic tree was constructed using Neighborhood Joining method.

2.5. Expression analysis of bu-lysin mRNA

The inducibility as well as the expression of bu-lysin in different tissues was studied using semi-quantitative RT PCR (Sundaresan et al., 2005).

2.5.1. Inducibility in ConA stimulated PBMC

Following the adjustment of concentration of PBMC to 5×10^6 cells per ml, the PBMC were cultured in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum (Sigma) and supplemented with 100 U Penicillin G and 100 μg Streptomycin per ml in 24-well tissue-culture plates in triplicates. PBMC were induced with ConA at final concentrations of 0, 5 and 10 μg per 5×10^6 cells. After 24 h of ConA induction, total RNA was isolated and was subsequently reverse transcribed as described above. Approximately 5 μl of 1:5 diluted first strand cDNA was

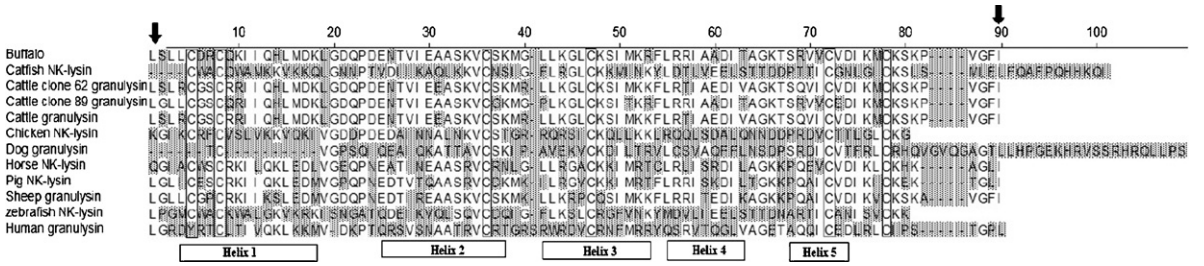

Fig. 1. Alignment of buffalo-lysin (EF583467), cattle clone 62 GNLY (AY245799), cattle clone 89 GNLY (AY245798), cattle GNLY (NM_001075143), sheep GNLY (EF654537), dog GNLY (XM_845424), human GNLY (NM006433), pig NK-lysin (X85431), horse NK-lysin (NM_001081929), chicken NK-lysin (DQ186291), catfish NK-lysin (AY934592) and zebra fish NK-lysin (AY184216). Complete conservation of six Cys residues in the mature peptide sequences are shown in box and arrows indicate the amino and carboxyl terminus of 9.2 kDD predicted mature bu-lysin peptide. Predicted helical regions of buffalo-lysin are based on human GNLY.

subjected to PCR using bovine lysin primers. To assess the variations in the efficiencies of the RT step among different experimental samples, β-actin (ACTB) was used as an internal control. A 190 bp fragment of ACTB was amplified independently using a pair of primers (Forward: 5′TACCCCATTGAGCACGGCAT3′ and Reverse: 5′ATGGCAGGGGTGTTGAAGGT3′). For amplification of ACTB gene, the same reaction conditions as described for the amplification of bu-lysin gene were used. Recombinant plasmid carrying bu-lysin gene was used as a positive control. In order to check the DNA contamination, for each RNA sample, a control reaction was set up in which reverse transcriptase enzyme was omitted during cDNA synthesis. The number of cycles used to amplify the cDNA was chosen to enable the PCR to proceed in a linear range in the preliminary experiments. Amplicons were analyzed on EtBr stained 1.5% agarose gel using gel-documentation system (Syngene, USA). The size and quantity of PCR products were verified by comparing with a low Range FastRuler^{TM} DNA Ladder, (Fermentas, USA). The concentrations of the ACTB mRNA and bu-lysin gene were determined using densitometry analysis as described by Sundaresan et al. (2005). Relative expression was determined as arbitrary units, defined as the ratio of mRNA level to the corresponding ACTB mRNA level after subtraction of background intensity [value = (intensity; gene of interest − intensity; background)/(intensity; ACTB − intensity; background)]. Mean values of the three measurements of each band from three independent samples were analyzed by paired *t*-test using Minitab (version 15.1.1.0; Minitab Inc., USA).

2.5.2. Expression in various tissues

RNA was also isolated from 100 mg of each tissue including spleen, kidney, liver, lymph node and uterine endometrium collected upon slaughter from apparently healthy adult buffaloes. Two micrograms of total RNA from each sample was reverse transcribed as described above. Three biological samples for each tissue were used in this study and values expressed as mean ± S.E.

3. Results and discussions

3.1. Cloning and characterization of bu-lysin

The cloned bu-lysin cDNA (GenBank Acc. No. EF583467) consists of 405 bp of open reading frame and encodes for the entire mature peptide of 84 amino acids with molecular weight of 9.2 kDD. GNLY/NKL is initially expressed as a 15 kDD non-lytic protein and subsequent post-translational processing of this protein yields a 9 kDD bioactive protein (Hanson et al., 1999). The multiple sequence alignment of nucleotide sequence of bu-lysin with those of other species (Fig. 1) revealed as high as 86–89% identity with the bovine lysin (clone 89 and 62), and 63, 47, 43, 24, 21, 58, 36, 26% identity with the GNLY/NKL homologue of bovine, porcine, equine, catfish, ovine, canine and human, respectively. The deduced bu-lysin mature peptide showed 85–91, 69, 18, 21, 55, 50, 22, 21, 19% identity with the bovine, ovine, canine, human, porcine, equine, chicken, catfish and zebra fish GNLY/NKL, respectively. Phylogenetic analysis of the nucleotide (not shown) and predicted mature peptide sequence (Fig. 2) of bu-lysin with that of GNLY/NKL of other species revealed the placement of bu-lysin in the same clad with the different polymorphic variants of bovine lysine. Even though the equine homologue possesses the same number of cationic amino acids as in bu-lysin, it fell into a separate clad with the porcine.

Multiple-alignment of the bu-lysin, various NKL and its homologue GNLY sequences indicates high-level secondary structure similarity (Fig. 1) having six conserved Cys residues and single Pro, which is the characteristic of the SAPLIP family (Andersson et al., 1995a). The number and placement of Cys primarily determines the bioactivity and stability of Cys-rich peptides. These Cys residues partici-

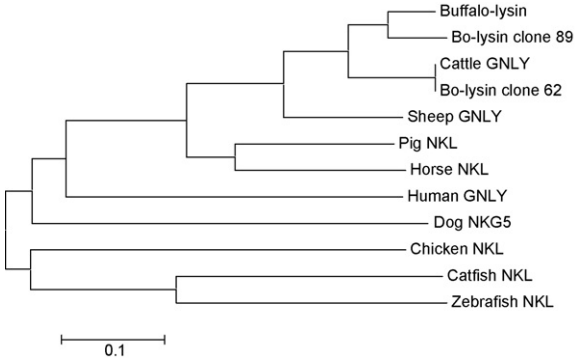

Fig. 2. Phylogram based on deduced amino acids sequences of buffalo and various species GNLY/NK-lysin.

pate in making disulfide bridges to form either multimeric bioactive molecule or to provide appropriate secondary structure required for bioactivities. However, the six Cys residues in GNLY/NKL including bu-lysin are conserved across the species indicating their importance for the antibacterial activity (Andersson et al., 1996; Wang et al., 2000).

The predicted 9 kDD mature peptide region of bu-lysin contains 19% of positively charged amino acids. However, mature peptide of bovine, equine, chicken, bovine and zebra fish GNLY/NKL possess 14, 21, 18, 14 and 12%, respectively. Cationic antimicrobial peptides generally have two to nine positively charged amino acids like Arg or Lys (Hancock and Diamond, 2000). These positively charged residues in the antimicrobial peptides are critical for lysis of both bacteria and tumor cells (Wang et al., 2000). The mature bu-lysin peptide sequence, similar to its equine homologue (Davis et al., 2005), has more number of (totally 11 residues, 13%) Lys residues than those in the bovine (Endsley et al., 2004) and porcine (Andersson et al., 1996) homologues, which have only seven (8%) or less lysine residues. However, the proportion and total number of Arg and Lys residues in the predicted antibacterial domain of bu-lysin (Table 1), helix 2 through helix 3 region, was conserved. Substitution of Arg with Lys in the cationic peptides is known to attenuate the antimicrobial activity (Wang et al., 2000). At the same time, replacements of Arg with Lys significantly reduce the cytotoxicity (Linde et al., 2005). In comparison with NKL/GNLY of different species, bu-lysin possesses higher proportion of Lys residue with the same total number of Arg. Consequently, bu-lysin might be speculated as a better effector antimicrobial peptide.

Table 1
Predicted antimicrobial region of NKL and GNLY of various species. Bold letters indicate arginine (**R**) and Italicized bold letters indicate lysine (**K**) residues.

Buffalo-lysin	VCS**K**-MGLL**K**GLC**K**SIM**KR**FL**RR**
Bovine-lysin clone 62	VCS**K**-MRLL**K**GLC**K**SIM**KK**FLRT
Bovine-lysin clone 89	VCG**K**-MGPL**K**GLC**K**SIT**KR**FL**RR**
Horse NK-lysin	VC**R**N-LGLL**R**GAC**KK**IM**R**TCL**R**L
Pig NK-lysin	VCD**K**-M**K**IL**R**GVC**KK**IM**R**TFL**RR**
Chicken NK-lysin	VCST-G**RR**Q**R**SIC**K**QLL**KK**L**R**QQ
Cattle granulysin	VCS**K**-M**R**LL**K**GLC**K**SIM**KK**FL**R**T
Sheep granulysin	VCS**K**-M**K**LL**KR**PCQSIM**KK**FL**RR**
Dog granulysin	VCS**K**-IPAVE**K**VC**K**DILT**R**VLCS
Human granulysin	VC**R**TG**R**S**R**W**R**DVC**R**NFM**RR**YQS**R**
Zebrafish NK-lysin	VCDQ-IGFL**K**SLCRGFVN**K**YMDV
Catfish NK-lysin	VCNS-IGFLRGLC**KK**MIN**K**YLDT

3.2. Expression profile of bu-lysin

In order to assess the inducibility of bu-lysin expression, PBMC was incubated with different concentrations of ConA. As shown in Fig. 3A and B, bu-lysin mRNA expression increased markedly ($p < 0.05$) in a dose dependant manner. Although GNLY/NKL is constitutively expressed, inducible activity is manifested particularly when T-lymphocytes are stimulated (Endsley et al., 2004).

Further, semi-quantitative-RTPCR analysis revealed differential expression of bu-lysin mRNA across the tissues (Fig. 3C and D). There was comparatively higher level of expression both in the spleen and lymph node, and moderate expression in the uterine endometrium. However, a very low level bu-lysin mRNA expression was observed in the liver and kidney. Since the lymph nodes and spleen are secondary lymphoid organs and known to have enriched lymphocyte population, relatively higher bu-lysin expres-

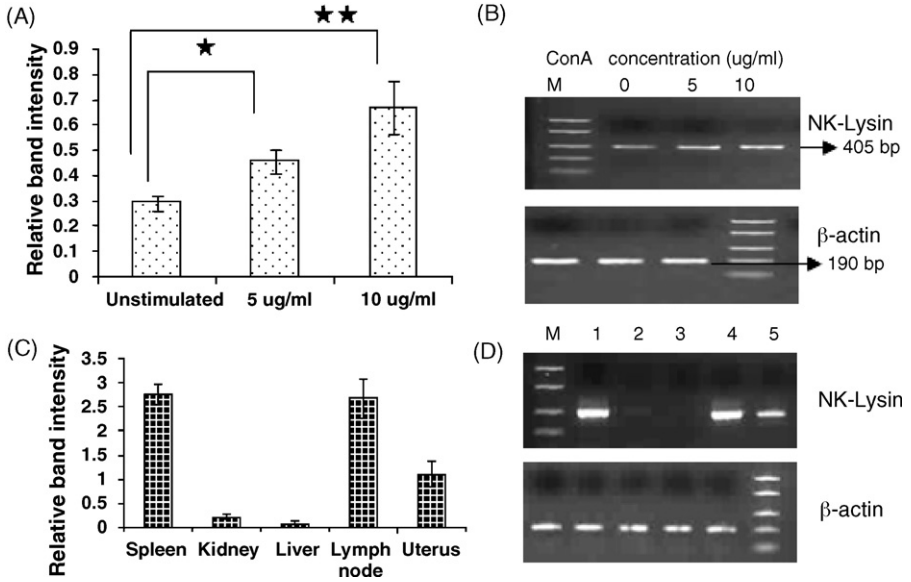

Fig. 3. Increased expression of buffalo-lysin gene in ConA stimulated PBMC. (A) Mean ratio of bu-lysin/beta actin relative expressions using semi-quantitative RT-PCR analysis (*$p < 0.04$; **$p < 0.02$). (B) Induction of bu-lysin gene expression in buffalo PBMC by various ConA doses (0, 5 and 10 μg/5×10^6 cells) from left to right lanes. (C) Bu-lysin gene expression profile analysis in different buffalo tissues. (D) RT-PCR amplified products of bu-lysin and beta-actin from different buffalo tissues viz., spleen (1), kidney (2), liver (3), lymph node (4) and uterus (5). The RT-PCR amplified bands of beta-actin and GNLY are indicated on the right margin.

sion in these tissues was observed. These findings are in conformity to the other studies in porcine (Andersson et al., 1995b) and equine (Davis et al., 2005). Another significant finding of this study is that a moderate level of bu-lysin expression was found in the secretory phase of buffalo endometrium (Fig. 3C). Higher NKG5 gene (a human GNLY homologue) expression was reported in human secretory endometrium and its expression was localized in the uterine NK-cells surrounding the glandular epithelium (Lobo et al., 2004). The uterine NK cell population increased during the secretory phase of human endometrium due to local proliferation and influx from peripheral circulation (Jones et al., 1997). So, we propose that buffalo endometrium is similar to human endometrium and may have uterine natural killer cell like lineage of cells and these cells might be contributing to the moderate level of bu-lysin expression. Further studies are needed to determine the cell lineage that expresses the bu-lysin and also localization of the cell lineage in the buffalo endometrium.

Identification of novel antimicrobial peptides from various species can be highly useful for development of alternative therapeutics to overcome the problems of antibiotic-resistance and the non-availability of newer classes of antibiotics. In food producing animals, knowledge about the presence of various antimicrobial peptides and their functions will be useful specifically in modulating immunity to combat various infectious diseases to augment productivity. While the antimicrobial activities of bu-lysin remain to be determined, our observations about the conservation of protein features and expression profiles suggested that bu-lysin is an important component of the buffalo immune mechanism.

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