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MOLECULAR CHARACTERIZATION OF β-LACTOGLOBULIN (βLG) GENE IN INDIAN BUFFALO (*Bubalus bubalis)*

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ABSTRACT

The aim of the study was to characterized tha β-lactoglobulin gene and protein in indian buffalo. The β-lactoglobulin (Bu_βLG) is a major whey milk protein and cause an allergic reaction in infants and children. In the present study, the full open reading frame (ORF) of buffalo β-lactoglobulin (Bu_βLG) gene was characterized, which consisted of a 543 bp sequence with 180 amino acid residues. The phylogenetic tree showed that the cattle, yak, and buffalo βLG gene formed one cluster and buffalo is more closer (96%) to an exotic cow (*Bos taurus*). ProtParam analysis showed that Bu_βLG protein was acidic (pI, 4.93), thermo-tolerant, and hydrophobic. The Bu_βLG 3-D model was generated by I-TASSER, which revealed a more stabilized nature of the predicted structure. Further, the Ramachandran plot validated that the 3-D model of Bu_βLG protein, which was of good quality. The presence of four ligand-binding sites for retinoic acid, oleic acid, vitamin D3, and benziphenone in Bu_βLG suggests that this protein bind to several fatty acids and ions. IEDB analysis displayed the seven and six epitope sites in the Bu_βLG protein for B-cell and T-cell, respectively. Thus, the epitope sites in the βLG protein may have certain immunological roles, which can be used to reduce the allergenicity of βLG protein for improving the buffalo milk quality.

Keywords: *Bubalus bubalis*, buffaloes, milk, β-lactoglobulin, mammary gland, Epithilial cells, protein structure

INTRODUCTION

Buffalo milk is highly nutritious and contains a higher amount of milk constituents compared to cow's milk. It is a rich source of vitamins, minerals, lipids, carbohydrates, and amino acids, which constitutes a complete food source for the neonates. The majority of the ruminants contain approximately 80% casein proteins (αS1, αS2, β, κ) and 20% whey proteins (β-lactoglobulin, α-lactalbumin, serum albumins) in the milk (Hoffman and Falvo, 2004). These constituents may vary across the breeds and among the individuals within the breed. In cattle, β-lactoglobulin (βLG or LGB) accounts for ~9% of the total milk protein (Ding *et al*., 2011). The bovine whey milk constitutes a mixture of βLG (~65%), LALBA (~25%), bovine serum albumin (~8%),

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several immunoglobulins, and other components (~2%) (Haug *et al*., 2007). However, buffalo milk contains less βLG as compared to indigenous cattle milk (Islam *et al*., 2014). On the other hand, human milk contains approximately 60% milk whey proteins and 40% casein proteins (Lonnerdal, 2003; Luhovyy *et al*., 2006). The human milk contains the caseins, α-lactalbumin, lactoferrin, immunoglobulin IgA, lysozyme, and serum albumins (Lonnerdal, 2004), but β-LG protein is absent in the human, lagomorph, and rodent milk (Sawyer and Kontopidis, 2000; Picariello *et al*., 2019).

The βLG is a major whey protein of cow, buffalo, sheep, goat, and non-ruminant species including pig, horse, dog, cat, dolphin, and marsupials (Sawyer and Kontopidis, 2000; Bawden *et al*., 1994). The βLG belongs to a lipocalins family of protein, which possesses a molecular pocket to accommodate iron complexes (Roth-Walter *et al*., 2014). However, the biological function of the βLG protein is not complete yet known, but it is believed that this protein binds to iron and play an important role in the transport of retinol and fatty acids (Kontopidis *et al*., 2002; Le *et al*., 2014). The βLG is also known as progestagen-associated endometrial protein (PAEP). It has been found that the βLG protein acts as an allergen and causes allergy after the ingestion of the milk (Selo *et al*., 1999; Kontopidis *et al*., 2004). In cattle, the βLG gene is located on chromosome 11 which consisted of 7 exons with ~6700bp long sequence (Otaviano *et al*., 2008**)**. Although, the βLG gene of cattle has several genetic variants *viz*. A, B, C, D, E, F, G, W, H, I, J and X, for which two isoforms namely, βLG A (β-LG A) and βLG B (β-LG B) were recognized in the milk (Aich *et al*., 2014). The A and B variants differ at position 64 codon (Asp/Gly) and 118 codons (Val/Ala) in the protein (Brownlow *et al*., 1997). Thus, the cattle βLG protein (mature portion) consisted of 162 amino acid residues (Le *et al*., 2014). On the other hand, the βLG gene is located on chromosome 12 in buffalo (Barłowska *et al*., 2012), but this gene is not completely characterized in this species.

The buffalo is a major milk producer in India and more than 50% of the milk is contributed by buffaloes. Nowadays, safety and quality assessment of milk used for human consumption has been considered as a major factor for improving the health of milk consumers. Therefore, the characterization of the whey protein gene (βLG) of buffalo is warranted to improve the milk quality aspects of buffalo. Very scanty literature is available on the structural characterization of the βLG gene in Indian buffalo. Therefore, the present study was undertaken to characterize the nucleotide and protein sequence and analyze major physiological properties such as α-helices, β-turn, different motifs, 3-D structure, and similarity to other proteins, post-translational modification, and antigenic behavior of the βLG protein in Indian buffalo.

MATERIALS AND METHODS

Isolation and culture of buffalo mammary epithelial cells

Buffalo mammary gland tissue was obtained from the slaughterhouse (Ghazipur, New Delhi, India) for isolation of the buffalo mammary epithelial cells (Bu-MECs). The same protocol was used as described by Anand *et al*. (2012) for the isolation and culture of the Bu-MECs with minor modifications. The Bu-MECs cell were cultured (Figure 1) in DMEM/F12 medium supplemented

with 1 µg/ml hydrocortisone (Sigma, USA), 10 ng/ml EGF (Sigma, USA), 10% FBS, 100 U/ml penicillin and 5 µg/ml streptomycin.

RNA isolation and synthesis of cDNA

Total RNA was prepared from buffalo mammary tissue and BuMECs using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. The possible genomic DNA contamination in RNA preparation was removed by DNA Inactivation Reagent (Ambion, USA). RNA integrity was assessed in 1.0% agarose gel by observing the rRNA bands corresponding to 28S, 18S, and 5.8S. The purity of RNA was checked through a nanodrop spectrophotometer with the ratio of optical density (OD) at 260 nm and 280 nm being >1.9. Complementary DNA was prepared using a RevertAid first-strand cDNA synthesis kit (Thermo Scientific, USA). Briefly, 10 ng RNA was reverse transcribed using 1 µL of M-MuLV reverse transcriptase (200 U/µL), 1 µL of RiboLock (20 U/µL), 2 µL of 10 mM dNTPs mix, 1 µL oligo dT primer, and 4 µL of 5X reaction buffer in 20 µL reaction volume. The cDNA was quantified by using nanodrop and the quality of cDNA was ascertained by agarose gel electrophoresis using GAPDH primers. The cDNA was stored at -20°C till further use.

Primer designing and PCR amplification

The primers for buffalo βLG were designed using Primer3 software based on the conserved sequences of cattle, sheep, and goats through multiple sequence alignment. These primer pairs (βLG forward: 5'-CCATGAAGTGCCTCCTGCT-3' and βLG reverse: 5'-ACGCCTTTATTGCTGAAGGA-3') were designed to flank the full coding region (open reading frame) of the βLG gene. The βLG gene of buffalo was amplified using gene-specific primers in Gene Pro Thermal Cycler (BIOER, China). The PCR reaction components were as follows; 1x PCR buffer, 1.66 U Taq DNA polymerase (5 U/µl), 1.5 mM $MgCl_2$, 250 µM dNTPs mix (10 mM), 4 µl cDNA, and 0.5 µM of each forward and reverse primer in 25 µl reaction volume. The PCR amplification reaction conditions were as follows: initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C for 45 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes.

Molecular cloning and sequencing

The PCR product of the βLG gene was purified by using a QIAquick Gel Extraction Kit (QIAGEN) and cloned in pJET1.2 blunt cloning vector (Thermo Scientific, USA). The PCR product with 3′-dA overhangs was blunted with a thermostable DNA blunting enzyme and then ligated to the linearized pJET1.2 blunt cloning vector as per the manufacturer instructions. The recombinant plasmid DNA was transformed into Top 10 *E. coli* competent cells. This vector contains a lethal protein gene that is disrupted by the ligation of DNA insert into a cloning site. As a result, only bacterial cells with recombinant plasmids can form the colonies. The single transformed colony was streaked on LB agar plate and subjected to colony PCR using a gene-specific primer with the same PCR reaction and amplification conditions. The amplified PCR products were checked on 1.5% agarose gel. The representative bacterial colonies showing the positive PCR bands were transferred into LB broth for the preparation of the plasmid DNA. The plasmid DNA was purified by using QIAprep Spin Miniprep Kit (QIAGEN). The purified plasmid DNA was sequenced through

Sanger sequencing.

Sequence analysis

The acquired buffalo βLG (Bu_βLG) gene sequence was submitted and the Gene Accession number *viz*. MT475827 was received from NCBI. The nucleotide sequence was assessed for their identity in comparison to the publicly available database in NCBI BLASTN (https://www.ncbi.nlm.nih.gov/BLAST). The sequence was further analyzed through the Bio-Edit tool. After analysis, the Bu_βLG gene sequence was translated into a protein sequence by using a program of 'Sequence Manipulation Suite' (www.bioinformatics.org). Phylogenetic analysis of the Bu_βLG gene was performed by using MEGAX software (version 10.1.5) to determine the evolutionary relationship between different closely related species.

Primary structure of Bu_βLG protein

The physicochemical characterization of the protein sequence of the Bu_βLG gene was analyzed by using the ExPASy-ProtParam tool (http://web.expasy.org/protparam) (Gasteiger *et al*., 2003), which computes the number of amino acids and its composition, theoretical isoelectric point (pI), molecular weight, grand average of hydropathicity (GRAVY), instability index, and aliphatic index.

Prediction of the secondary structure of Bu_βLG protein

The secondary structure of Bu_βLG protein sequence was examined through the SOPMA (Self-Optimized Prediction Method with Alignment) server (Geourjon and Deleage, 1995), which computes the percentage of α-helices, β turn, and β-sheet in the protein.

Homology modeling and validation of Bu_βLG protein

The three-dimensional structure of Bu_βLG protein was predicted through the I-TASSER (Iterative Threading ASSEmbly Refinement) server. This is a hierarchical approach to predict the protein structure by an alignment of the structural template retrieved from the protein data bank (PDB) and generate the full-length atomic models by assembling the repetitive template fragment. I-TASSER predicts the structure based on the confidence score (C-score) and template modeling score (TM-score). The C-score is used to determine the quality of the predicted model and this score normally ranges from (-5, 2), where a higher value signifies a model with high confidence level and vice-versa. TM-score is used to determine the structural similarity between the two structures. It measures the accuracy of structure modeling when a native structure is known. A TM score >0.5 indicates that the predicted model is of correct topology and a TM score <0.17 explains the random similarity. I-TASSER also acknowledges the normalized B-factor for structure prediction. B-factor (also known as temperature factor) is used to estimate the extent of atomic motion in the X-ray crystallography experiment. The more negative value of the B-factor reveals more stability of protein structure (Zhang, 2008). To validate the I-TASSER model, we predicted the Bu_βLG protein models through SWISS-MODEL software (Waterhouse *et al*., 2018), which produce the three-dimensional structures based on the homology template from the protein data bank (PDB). Further, the predicted structure of Bu_βLG protein was validated through the construction of Ramachandran plot by PROCHECK server (Laskowski *et al*., 1993) to visualize the energetically allowed regions for

backbone dihedral angles ψ against φ of the amino acid residues of protein structure.

Prediction of immunological sites

Immunological sites play a significant role in determining the antigenic nature of the protein (Larsen *et al*., 2006). The B-cell and T-cell linear epitopes for Bu_βLG protein was predicted through the IEDB tool server (Larsen *et al*., 2006; Paul *et al*., 2014).

RESULT AND DISCUSSION

Sequence analysis of the full coding region of Bu_BLG gene

The full ORF (open reading frame) of Bu_βLG gene was amplified and the resulting PCR product was 739 bp long (Figure 2). The product was cloned and sequenced through Sangar sequencing. The analysis showed that Bu_βLG ORF consisted of 543 bp (corresponding to 180 amino acid residues). However, one nucleotide change in the coding sequence of the Bu_βLG gene was observed at 486 bp position (CCC to CCT) (Figure 3) as compared to earlier published buffalo βLG sequence, but no change in the amino acid sequence was observed (Figure 4). The multiple sequence alignment (MSA) of the Bu_βLG protein was carried out with other species such as wild yak, exotic cow, indigenous cow, sheep, goat, and horse as shown in Figure 4. It was shown that the Bu_βLG sequence was more different from the cattle and horse βLG sequences (Figure 4). The cattle βLG ORF was consisted of 537 bp long with a deletion of 6 bp (which codes for two amino acid residues) as shown in NCBI published sequences (Acc. No. NM_173929.3; XM_019971082.1; XM_027556707.1; EU883598.1) compared to buffalo βLG ORF sequence (Figure 4). Further, the number of amino acid residues in Bu_βLG protein (mature protein: 161aa) was different from the cattle βLG protein (mature protein: 162aa). It is indicated that the buffalo βLG amino acid sequence is slightly different from the cattle but more variable from the horse βLG sequence.

Sequence homology and phylogenetic relationship

The acquired nucleotide and translated amino acid sequence of Bu_βLG were analyzed for sequence homology through NCBI BLASTN and BLASTP tools. The acquired nucleotide sequence of the Bu_βLG gene showed 99.82% identity with the published βLG mRNA sequence of the buffalo. The translated amino acid sequence of the gene showed higher similarity with the βLG protein sequence of the published buffalo sequence as shown in GenBank. Further, βLG nucleotide sequence similarities with other species were checked through the BLAST search tool. The Bu_βLG mRNA sequence similarity with the Wild yak (*Bos mutus*), Exotic cow (*Bos taurus*), Indian cow (*Bos indicus*), Goat (*Capra hircus*), Sheep (*Ovis areas*) and Horse (*Equus caballus*) was about 96.69%, 96.50%, 96.32%, 96.32%, 95.95%, and 80.04%, respectively. It is concluded that the Bu_βLG has more similarities with Wild yak in the study.

Phylogenetic tree of buffalo βLG gene was constructed against the βLG nucleotide sequences of Cattle (*Bos taurus and Bos indicus),* Sheep (*Ovis aries*), Goat (*Capra hircus*), Yak (*Bos grunniens*), and Horse (*Equus caballus*) with the help of MEGAX (version 10.1.5) program. The phylogenetic tree (Figure 5) constructed based on the βLG gene suggested that the cattle, yak, and buffalo βLG gene formed a cluster with 94%

similarity and the buffalo was more closer (96%) to an exotic cow (*Bos taurus*). While sheep and goat βLG formed another cluster (99%) with a close relationship with each other. However, the horse βLG gene was placed as an out-group in a tree. The results of the present study indicated that Bu_βLG sequence has more recumbences with exotic cow and domestic yak sequences; therefore it is presumed that the buffalo milk protein may have more similarity with cattle and yak rather than the sheep, goat, and horse.

Evaluation of primary structure of Bu_βLG

The primary structure of Bu_βLG protein was evaluated by certain physicochemical parameters such as the number of amino acids, molecular weight, iso-electric point (pI), instability index, aliphatic index, and grand average of hydropathicity (GRAVY) as shown in Table 1. The full Bu_βLG protein contains the 180 amino acid residues, while the mature Bu_βLG protein contains the 161 amino acid residues with 19 amino acid residues as a signal sequence. The molecular weight of Bu_βLG protein has been shown in Table 1. It is well known that the high pI value represents the basic nature of the amino acids, while low pI value indicates the acidic nature of the amino acids. The result of ExPASy-ProtParam analysis showed that the pI value of Bu_βLG protein was acidic (pI, 4.93), while pI of bovine βLG protein was slightly higher (pI, 5.1) (Verheul *et al*., 1999) as found in the current study. It is well known that the stability of a protein is determined by the instability index. If the instability index value is <40, then the protein will be stable; and if the value is >40 then the protein will be unstable. In this context, our data indicated that the Bu_βLG protein is less stable as the instability index is close to 40 (Table 1). The aliphatic index of the protein was analyzed and this index was based on the presence of aliphatic amino acids (alanine, valine, isoleucine, and leucine) residing in the aliphatic side chain of that protein. A higher value of an aliphatic index indicates the thermo-tolerance nature of the protein (Filiz and Koc, 2014). In this study, the Bu_βLG protein contains a comparatively high percentage of aliphatic amino acids showing the thermostable nature of the proteins. However, an aliphatic index of the Bu_βLG protein was higher (107.39), which advocated that this protein is more thermo-tolerant (Table 1). The GRAVY value specified the hydrophilic and hydrophobic nature of the protein (Filiz and Koc, 2014). In the present study, a positive GRAVY value (0.026) was found for Bu_βLG protein, which proposed the hydrophobic nature of this protein (Table 1). It is well proved that the native bovine βLG protein binds to several ligand molecules such as retinol and fatty acids which further verify that the cattle βLG is hydrophobic nature as evidenced by several workers (Kontopidis *et al*., 2002; Puyol *et al*., 1993). It is concluded that Bu_βLG is more basic, hydrophobic, and thermo-tolerant.

Evaluation of the secondary structure of Bu_βLG

The secondary structure of Bu_βLG protein was predicted by the SOPMA server, which is shown in Figure 6. The secondary structure of the protein is stabilized by hydrophobic, ionic, and hydrogen bond interactions between the peptide chains. Protein flexibility is defined by the presence of more random coils (Berjanskii and Wishart, 2008). In the present study, the low percentage of random coils was observed in Bu_βLG (27.78%) which confirmed that this protein was slightly rigid and less flexible (Figure 6). Similarly, the high percentage of α-helix was

found in Bu_βLG (48.33%) which suggests that the protein was thermostable, since the thermophilic nature of protein has more abundance of alpha-helices as suggested by previous workers (Kumar *et al*., 2000). It has been reported that the bovine βLG structure consisted of nine β-strands labeled from A to I, which forms two β-sheets, and three turns of α-helix (Le *et al*., 2014). The earlier studies showed that bovine βLG protein comprised of 15% α-helix, 50% β-sheet, and 15 to 20% reverse turn (Sawyer and Kontopidis, 2000; Kuwata *et al*., 1998). Previous studies have shown that the β-sheet of bovine βLG protein contains a central β-barrel which is formed by eight antiparallel β-strands (β_A-β_H), an additional β-strand (β_I), one major *α*-helix, and four short *α*-helices (Brownlow *et al*., 1997; Kuwata *et al*., 1998). Further research showed that the protein had a barrel structure by eight antiparallel β-strands and α-helix located at the outer surface of the barrel (Liang *et al*., 2008). The bovine βLG protein existed in the dimeric form at neutral pH 7 (Verheul *et al*., 1999), while it dissociates into monomeric form at pH 3.5 and retains its native conformation at low pH ~2 [18]. Thus, the βLG is generally monomeric at low pH 3 (Verheul *et al*., 1999) and more stable at low pH ~3 (Crowther *et al*., 2016). So, under physiological conditions, βLG protein is in equilibrium between monomers and dimers. However, this equilibrium may be affected by protein concentration, pH, ionic strength, and temperature (Aymard *et al*., 1996; Renard *et al*., 1998; Mercadante *et al*., 2012).

In the present study, the percentage of α-helix was calculated based on the full coding of buffalo βLG protein, which was higher than the α-helix of the βLG protein of cattle. Further analysis showed that the percentage of α-helix in mature Bu_βLG (40.99%) protein was higher than that of mature βLG protein of cattle. The random coil in the full and mature form of Bu_βLG protein was observed as 27.78% and 35.40%, respectively (Figure 6). It is concluded from the above discussion that the buffalo βLG protein has more percentage of α-helix than the cattle βLG protein.

Prediction of tertiary structure and its validation

The 3D structure of Bu_βLG protein was predicted through the I-TASSER server. The 3-D Model-1 was selected for Bu_βLG protein (Figure 7), which has the C-score and TM-score as -0.54 and 0.64, respectively. Thus, Bu_βLG contains about 50 coils, 35 strands, and 87 helixes and has a negative peak of normalized B-factor, showing more stability of the predicted structure. An earlier study has shown that the 3-D structure of the βLG protein consisted of anti-parallel β-sheet, and has eight anti-parallel β-barrels (A-H) (Papiz *et al*., 1986). The strands A-D form one sheet and strands E-H form a second sheet. The loops that are linked to the β-strands at the closed end of the calyx, BC, DE, and FG are short, while the loop at the open end is slightly longer and more flexible in bovine βLG as shown in published work (Kontopidis *et al*., 2004). It was suggested that the stability of the βLG protein was governed by a change of Ala^{118} to Val^{118}, which was caused by differences in the thermal aggregation as suggested by earlier workers (Brownlow *et al*., 1997). Further, the validation of stereochemistry of predicted Bu_βLG protein tertiary structure was done by Ramachandran plot using the PROCHECK server. It was found that the Ramachandran plot of Bu_βLG protein contains 89% residues in the most favored region and 9.1% residues in an additional allowed region (Figure 7) indicating that this protein model was of good quality. It has been demonstrated that βLG protein binds to numerous hydrophobic ligands such as

retinol, vitamin D, cholesterol, fatty acid, and their derivatives, and aromatic compounds, however, its biological function is still unknown (Le *et al*., 2014). The main binding site of βLG for hydrophobic ligands was formed by the calyx of βLG protein, which contains Glu^{62} and Lys^{69} at the entrance of the calyx (Loch *et al*., 2013). In our predicted βLG protein model 1, four potential ligand-binding sites were found namely retinoic acid (binding site residues: 57,59,74,78,80,87,89,110,123,125,136,138), Oleic acid (binding site residues: 59,64,72,74,78,80,89,102,110,112,121,123,125), Vitamin D3 (binding site residues: 56,57,59,61,74,78,80,89,102,104,123,125), and Benziphenone (binding site residues: 57,61,102,110,123,125,136,140). It is very difficult to compare and discuss the structural features of Bu_BLG protein with other species. The different species may have a variable number of amino acid sequence and composition and most of the parameters would be different among the species. It was proposed that the crystal structure of ovine (Loch *et al*., 2014; Kontopidis *et al*., 2014) and caprine (Crowther *et al*., 2014) βLG protein shared a high degree of structural similarity with bovine βLG at the level of tertiary and quaternary structure (Crowther *et al*., 2016). It is summarized that the milk protein (βLG) binds to several fatty acids, ions, ligand molecules and acts as a carrier and transport protein for various cell signaling processes in buffalo, however, the structure difference may be due to the amino acid compositions and power of the bioinformatics tools and software used in the analysis of the tertiary structures by previous workers.

Prediction of immunological sites

In the present study, IEDB analysis showed that seven potential immunological sites were predicted in Bu_βLG protein for B-cell (Table 2), which can act as an antigen. Further, six epitopic sites were predicted in βLG protein for T-cell (Table 3). It is well known that βLG β-sheet contains nine antiparallel β-strands from A to I (Jameson *et al*., 2002), while an epitope was located within the D strand of β-LG (residues 66 to 76) and the reactive site was associated with AQKKIIAEK sequence of β-LG (Song *et al*., 2005). Six to eight IgE binding sites were identified on the βLG molecule by several workers (Selo *et al*., 1999; Ball *et al*., 1994; Niemi *et al*., 2007). Apart from the B-cell epitopes, three dominant T cell epitopes were identified on βLG protein in mice (Totsuka *et al*., 1997). A subsequent study (Inoue *et al*., 2001) demonstrated that the seven T cell epitopes were found on different human βLG peptides. The results of the analysis indicated that the Bu_βLG has immunological epitopic sites, which can be used further for the generation of antibodies *in vivo* against these epitopes to reduce the immunoreactivity of these proteins for the safety aspect of the milk (Batra *et al*., 2019).

CONCLUSION

In the present study, buffalo βLG nucleotide and protein sequence were characterized. This study showed one allelic variation in the Bu_βLG gene, which may occur due to unknown factors or mutation. The Bu_βLG sequence was different from the βLG sequence of cattle and the horse. The phylogenetic tree constructed based on the βLG sequence data showed that the cattle, yak, and buffalo formed a cluster with a closer relationship between exotic cow and buffalo followed by sheep and goat group. Computational analysis showed that the Bu_βLG protein was slightly thermostable, acidic, and hydrophobic based on the

Table 1. Physicochemical characteristics of the Bu_BLG protein.

Physicochemical	Bu_BLG parameters
Number of amino acids	180
Theoretical pI	4.93
Molecular weight	20009.41
Instabiliy index	39.79
Aliphatic index	107.39
Grand average of hydropathicity (GRAVY)	0.026
Total number of negatively charged residues (Asp + Glu)	26
Total number of positively charged residues (Arg + Lys)	19

Table 2. Different B-cell epitopic sites in Bu_BLG protein.

Site No.	Start	End	Peptide	Length
1	54	55	SA	2
2	64	71	LKPTPEGD	8
3	80	85	ENGECA	6
4	95	95	K	1
5	128	134	SAEPEQS	7
6	144	152	PEVDDEALE	9
7	172	175	TQLE	4

Table 3. Different T-cell epitopic sites in Bu_BLG protein.

Site No.	Start	End	Peptide	Immunogenicity score
1	46	60	DISLLDAQSAPLRVY	98.1148
2	36	50	TWYSLAMAASDISLL	96.3553
3	16	30	AQAIIVTQTMKGLDI	93.0655
4	161	175	LPMHIRLSFNPTQLE	91.7711
5	96	110	IPAVFKIDALNENKV	84.916
6	151	165	LEKFDKALKALPMHI	82.7607

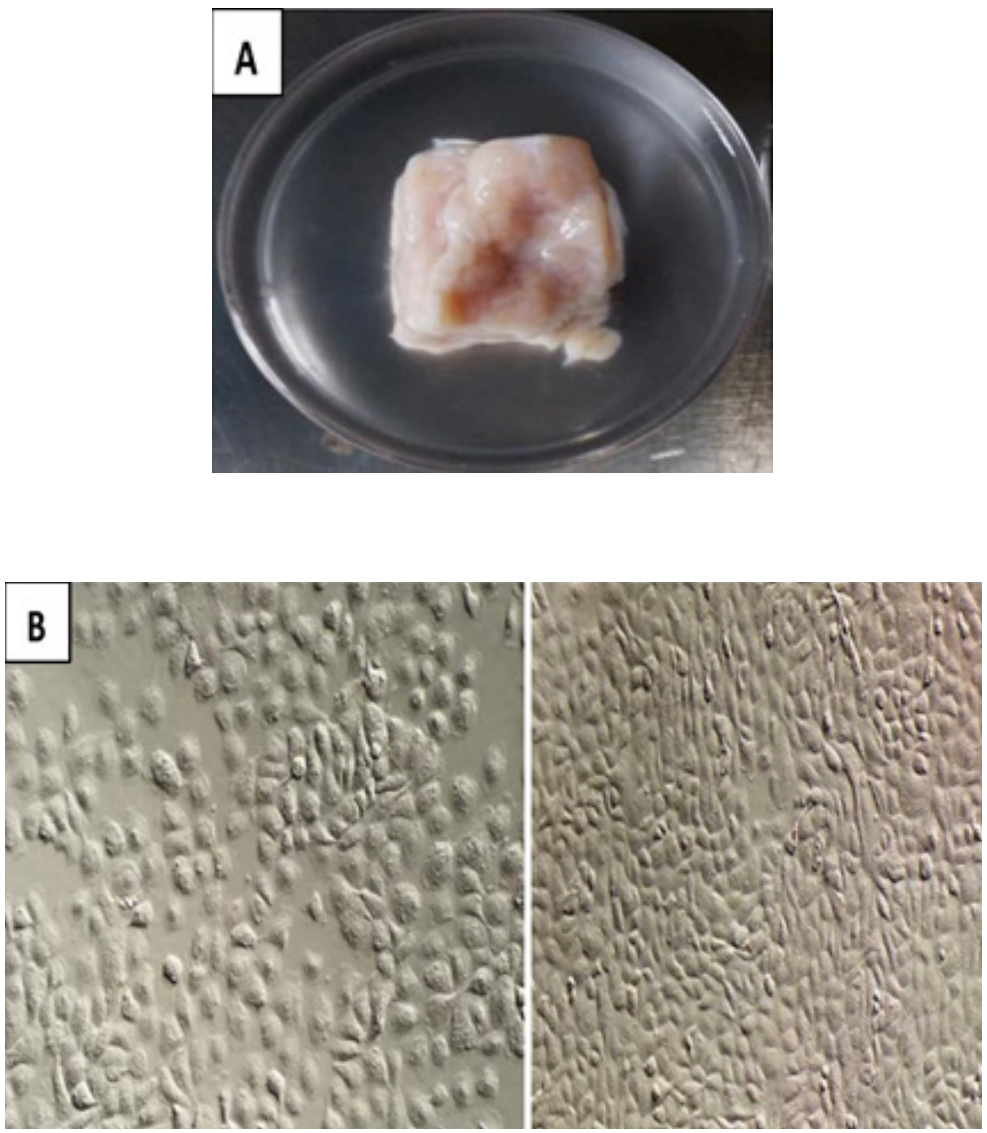

Figure 1. (A) Buffalo mammary gland tissue; (B) Cultured buffalo mammary epithelial cells (BuMEC).

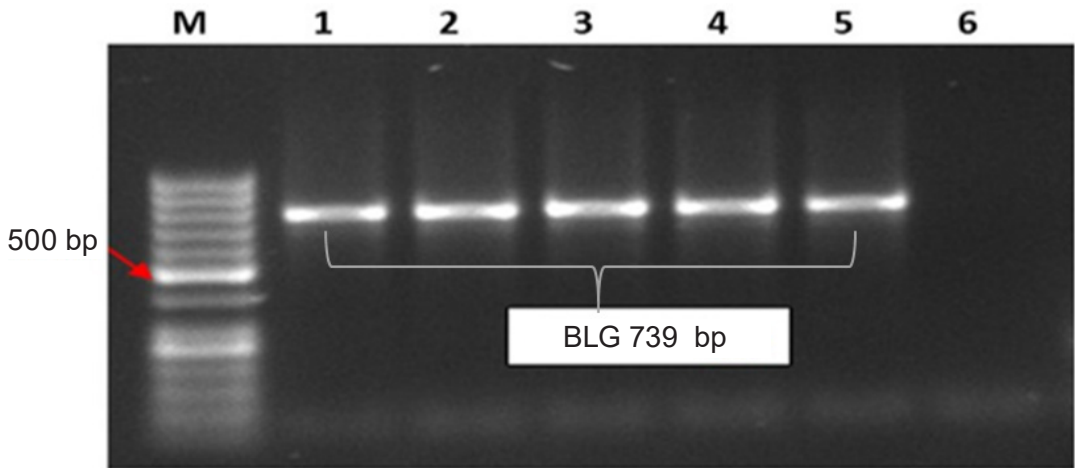

Figure 2. Agarose gel electrophoresis of Bu_βLG PCR products using a gene-specific primer. Lane 1-6: Bu_βLG samples; M: 50 bp DNA ladder.

> β-LG _FULL CODING NUCLEOTIDE SEQUENCE OF BUFFALO (MT475827)

ATGAAGTGCCTCCTGCTTGCCCTGGGCCTGGCCCTTGCCTGTGGCGCCCAGGCCATCATCGTCACCCAGACCATGAAGGGC
CTGGACATCCAGAAGGTGGCGGGGACTTGGTACTCCTTGGCCATGGCGGCCAGCGACATCTCCCTGCTGGACGCCCAGAG
TGCCCCCCTGAGAGTGTATGTGGAGGAGCTGAAGCCCACCCCTGAGGGCGACCTGGAGATCCTGCTGCAGAAATGGGAG
AATGGTGAGTGTGCTCAGAAGAAGATCATTGCAGAAAAAACCAAGATCCCTGCCGTGTTCAAGATCGACGCCTTGAACGA
GAACAAAGTCCTTGTGCTGGACACCGACTACAAAAAGTACCTGCTCTTCTGCATGGAGAACAGTGCTGAGCCCGAGCAAA
GCCTGGCCTGCCAGTGCCTGGTCAGGACCCCGGAGGTGGACGACGAGGCCCTGGAGAAATTTGACAAAGCCCTCAAGGC
CCTGCCTATGCACATCCGGCTCTCCTTCAACCCGACCCAGCTGGAGGAGCAGTGCCACGTC**TAG**

Figure 3. The full coding nucleotide sequence of βLG gene in buffalo.

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                                10        20        30        40        50        60
                         ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Bubalus bubalis (MT475827)      MKCLLLALGL ALACGAQAII VTQTMKGLDI QKVAGTWYSL AMAASDISLL DAQSAPLRVY
Bubalus bubalis (NM_001290964.  MKCLLLALGL ALACGAQAII VTQTMKGLDI QKVAGTWYSL AMAASDISLL DAQSAPLRVY
Bos mutus (XM_014481456.1)      MKCLLLAL-- ALTCGAQALI VTQTMKGLDI QKVAGTWYSL AMAASDISLL DAQSAPLRVY
Bos taurus (EU883598.1)         MKCLLLAL-- ALTCGAQALI VTQTMKGLDI QKVAGTWYSL AMAASDISLL DAQSAPLRVY
Bos indicus (XM_027556707.1)    MKCLLLAL-- ALTCGAQALI VTQTMKGLDI QKVAGTWYSL AMAASDISLL DAQSAPLRVY
Capra hircus (NM_001285539.1)   MKCLLLALGL ALACGIQAII VTQTMKGLDI QKVAGTWYSL AMAASDISLL DAQSAPLRVY
Ovis aries (NM_001009366.1)     MKCLLLALGL ALACGVQAII VTQTMKGLDI QKVAGTWHSL AMAASDISLL DAQSAPLRVY
Equus caballus (NM_001082493.3  MKCLLLALGL ALMCGIQATN IPQTMQDLDL QEVAGKWHSV AMAASDISLL DSESAPLRVY
Clustal Consensus               ******** ** ** **  :.***:.**: *:***.*:*: ********** *::*******

                                70        80        90       100       110       120
                         ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Bubalus bubalis (MT475827)      VEELKPTPEG DLEILLQKWE NGECAQKKII AEKTKIPAVF KIDALNENKV LVLDTDYKKY
Bubalus bubalis (NM_001290964.  VEELKPTPEG DLEILLQKWE NGECAQKKII AEKTKIPAVF KIDALNENKV LVLDTDYKKY
Bos mutus (XM_014481456.1)      VEELKPTPEG DLEILLQKWE NGECAQKKII AEKTKIPAVF KIDALNENKV LVLDTDYKKY
Bos taurus (EU883598.1)         VEELKPTPEG DLEILLQKWE NDECAQKKII AEKTKIPAVF KIDALNENKV LVLDTDYKKY
Bos indicus (XM_027556707.1)    VEELKPTPEG DLEILLQKWE NGECAQKKII AEKTKIPAVF KIDALNENKV LVLDTDYKKY
Capra hircus (NM_001285539.1)   VEELKPTPEG NLEILLQKWE NGECAQKKII AEKTKIPAVF KIDALNENKV LVLDTDYKKY
Ovis aries (NM_001009366.1)     VEELKPTPEG NLEILLQKWE NGECAQKKII AEKTKIPAVF KIDALNENKV LVLDTDYKKY
Equus caballus (NM_001082493.3  IEKLRPTPED NLEIILREGE NKGCAEKKIF AEKTESPAEF KINYLDEDTV FALDTDYKNY
Clustal Consensus               :*:*:****. :***:*:: * *  **:***: ****: ** * **: *:*:.* :.******:*

                               130       140       150       160       170       180
                         ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Bubalus bubalis (MT475827)      LLFCMENSAE PEQSLACQCL VRTPEVDDEA LEKFDKALKA LPMHIRLSFN PTQLEEQCHV
Bubalus bubalis (NM_001290964.  LLFCMENSAE PEQSLACQCL VRTPEVDDEA LEKFDKALKA LPMHIRLSFN PTQLEEQCHV
Bos mutus (XM_014481456.1)      LLFCMENSAE PEQSLACQCL VRTPEVDDEA LEKFDKALKA LPMHIRLSFN PTQLEGQCHI
Bos taurus (EU883598.1)         LLFCMENSAE PEQSLVCQCL VRTPEVDDEA LEKFDKALKA LPMHIRLSFN PTQLEEQCHI
Bos indicus (XM_027556707.1)    LLFCMENSAE PEQSLACQCL VRTPEVDDEA LEKFDKALKA LPMHIRLSFN PTQLEEQCHI
Capra hircus (NM_001285539.1)   LLFCMENSAE PEQSLACQCL VRTPEVDKEA LEKFDKALKA LPMHIRLAFN PTQLEGQCHV
Ovis aries (NM_001009366.1)     LLFCMENSAE PEQSLACQCL VRTPEVDNEA LEKFDKALKA LPMHIRLAFN PTQLEGQCHV
Equus caballus (NM_001082493.3  LFLCMKNAAT PGQSLVCQYL ARTQMVDEEI MEKFRRALQP LPGRVQIVPD LTRMAERCRI
Clustal Consensus               *::**:*:*  * ***.** * .**  **.*  :*** :**:. ** ::::  :  *::  :*::
```

Figure 4. Multiple sequence alignment of the amino acid sequence of Bu_βLG protein (180aa) with βLG sequences of wild yak, exotic cow, indigenous cow, sheep, goat, and horse through Bio-Edit tools.

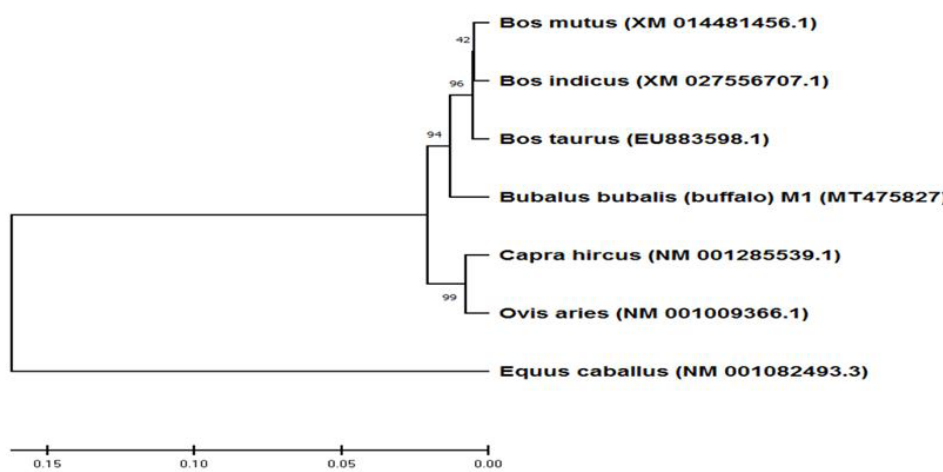

Figure 5. Phylogenetic tree showing the evolutionary relationship of buffalo with different species based on the nucleotide sequences of the β-lactoglobulin gene from other species. The analysis was carried out using the UPGMA method with bootstrap values from 1000 replicates, as indicated by the numerical values on the nodes.

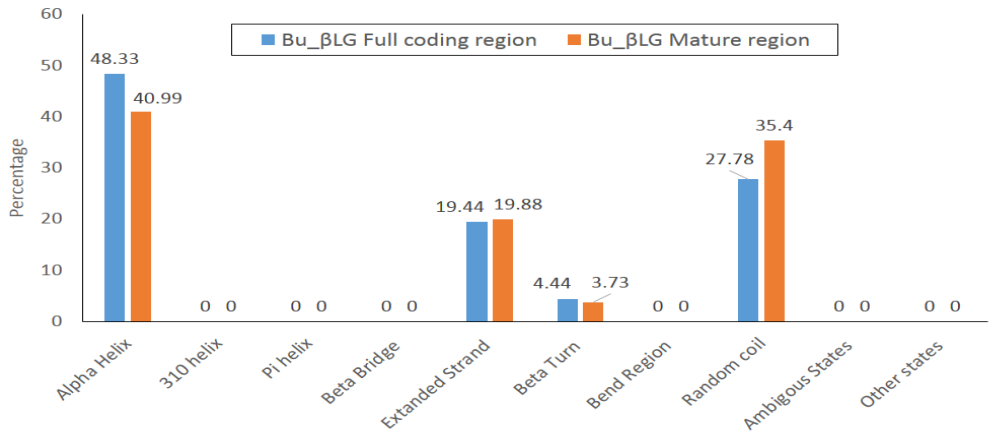

Figure 6. Graphical representation of the analysis of the secondary structure of full coding and the mature region of Bu_βLG protein using the SOPMA server.

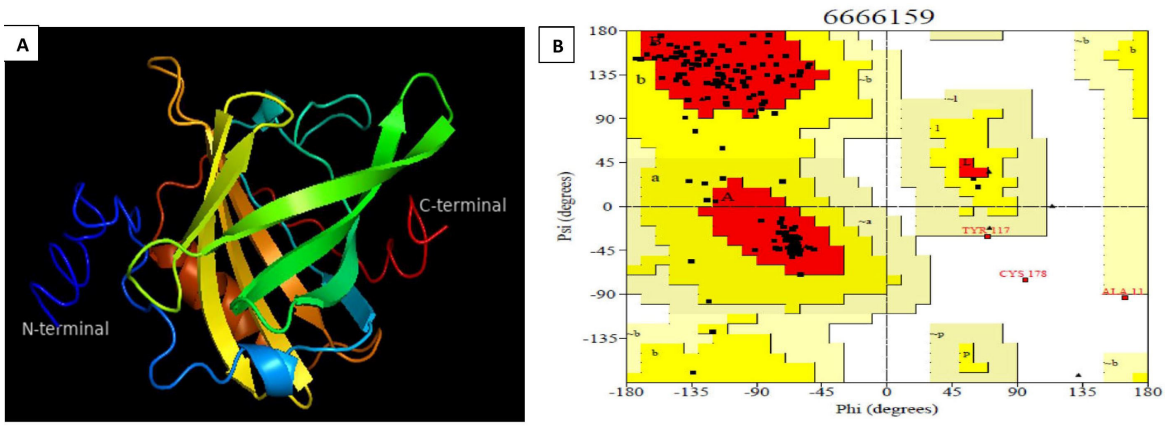

Figure 7. (A) Predicted 3D structure of the Bu_βLG protein by I-TASSER Server; (B) Ramachandran plot of Bu_βLG protein generated by PROCHECK server (White-Region 0: Disallowed; Cream-Region 1: Generous; Yellow-Region 2: Allowed; Red-Region 3: Most favorable core region; Black Dot-Colour of markers in favorable regions; Red DOT-Colour of markers in unfavorable regions).

hydropathicity index. It was found that the βLG protein has a less flexible protein structure, but the presence of motifs in the proteins play a key role in various cell signaling processes. Thus, the presence of different immunological epitope sites on Bu_βLG protein proved that this protein may act as an antigen and may elicit the antibody responses.

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