RESEARCH ARTICLE

Cloning and Characterization of α-S1-casein (*CSN1S1*) gene and Protein in Indian buffalo (*Bubalus bubalis*)

Vinay K. Mehra[1*], Dhruba Malakar[1], Satish Kumar[1*]

Abstract

In buffalo, casein genes are located on chromosome 7 (250 kb). The genetic variants of caseins influence the production and nutritional quality of milk. The bovine α s1-casein is up to 40 % of total bovine caseins in milk and is encoded by *CSN1S1* gene (~ 17.5 kb) and it is identified as allergens and responsible for cow's milk allergy in infants. *CSN1S1* gene was amplified using gene-specific primers, cloned into pJET1.2 cloning vector. The full ORF of buffalo *CSN1S1* gene was characterized, which consisted of 801 bp. The protein sequence analysis of buffalo *CSN1S1* gene showed that serine is replaced by leucine at 193 amino acid position. The negative GRAVY (grand average of hydropathy) value of αs1-casein protein indicated the hydrophilic nature and high percentage (44.86%) of random coils in confirmed flexible nature of the protein. NetPhos 3.1 analysis showed this protein was phosphorylated (17 residues), which may be involved in post-translational modification processes. IEDB (The Immune Epitope Database) analysis showed 3 potential immunological sites in α s1-casein for B-cell, which may involve certain immunological responses. The phylogenetic tree showed that cattle, yak and buffalo formed one cluster (95%) with a closer relationship between *Bos taurus and Bos indicus (99%)*. The buffalo *CSN1S1* gene is closer to yak *CSN1S1* gene (95%).

Keywords: *CSN1S1* gene, αs1-casein, Buffalo, Milk

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Introduction

Milk is a source of dietary energy and provides essential nutrients. In cattle, all the casein genes are located on chromosome 6 in cluster form, which occupies 200 Kb region. These genes are arranged in order: *CSN1S1*, *CSN2*, *CSN1S2*, *CSN3* and encode αs1-casein, αs2-casein, β-casein, and κ-casein protein respectively (Bai *et al.,* 2007). Similarly, in buffalo, casein loci are located on chromosome 7 and organized in a cluster of approximately 250 kb (Vinesh *et al.,* 2013). The four types of caseins αs1-casein, αs2-casein, β-casein and κ-casein contribute 20.61%, 14.28%, 53.45%, 11.66% of the total casein in milk, respectively (Barlowska *et al.,* 2012). Milk caseins show breed to breed and animal to animal variations in their quantities in milk and their polymorphism, and these genetic variants of caseins influence the production and nutritional quality of milk. The αs1- and αs2-caseins are calcium-sensitive, and they are precipitated in the presence of low concentrations of calcium (Misra *et al.,* 2008).

The primary function of αs1-casein is to export caseins from the endoplasmic reticulum (ER) and help in early casein micelle formation and casein transport in the secretory pathway (Parc et al., 2010). The αs1-casein is up to 40 % of total bovine caseins in milk (Barlowska *et al.,* 2012). The *CSN1S1* gene showed a relatively sizeable transcriptional unit of about 17.5 kb nucleotide (Ramunno *et al.,* 2004). Eight allelic variations (A, B, C, D, E, F, G, and H) of αs1-casein have been identified. Variant A found in *Holstein Friesians* (Farrell *et al.,* 2004). Variant B is predominant in *Bos taurus* and variant C in *Bos indicus* and *Bos grunniens* (Eigel *et al.,* 1984). Allele B at 192 positions of the polypeptide chain encodes glutathione, whereas the allele C encodes glycine (Barlowska *et al.,* 2012). In buffalo and cattle, the *CSN1S1* gene encodes 214 amino acids precursor with a signal peptide of 15 amino acid residues and a mature protein of 199 amino acids residues (Nahas *et al.,* 2013). In αs1-casein, there are two components: major and minor; both have the same amino acid sequence. They only differ by degree of phosphorylation, and in specific milk, genetic variants of both components are identical, but in the case of milk from heterozygous animals, two genetic variants are present (Eigel *et al.,* 1984). It has also been reported that αs1-casein is identified as allergens and responsible for cow's milk allergy in infants and shows IgE reactivity (Orru *et al.,* 2013). The objective of the present study was to characterize the

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buffalo *CSN1S1* gene and its protein at the molecular level and *in-silico* prediction of a possible secondary structure, physical properties, and immunogenicity nature of the αs1-casein protein.

Materials and Methods

Isolation and Culture of Buffalo Mammary Epithelial Cells

Buffalo mammary gland tissue was obtained from a local slaughterhouse (New Delhi, India) to isolate Buffalo Mammary Epithelial Cells (BuMEC). We followed essentially the same protocol used by Anand *et al*. (2012) to isolate BuMEC with minor modifications.

RNA Isolation and cDNA Synthesis

According to the manufacturer's protocol, total RNA from buffalo mammary tissue and BuMECs were prepared using TRIzol (Invitrogen, USA). RNA integrity was assessed in 1.5% agarose gel electrophoresis by observing rRNA bands of 28S and 18S. According to the manufacturer's protocol, possible genomic DNA contamination in RNA preparation was removed using a DNA-free kit (Ambion, USA). The Purity of RNA was checked in UV spectrometer with the ratio of the OD at 260 nm and 280 nm being >1.8. cDNA was synthesized by reverse transcription PCR using Revert Aid First strand cDNA synthesis kit (Thermo Scientific, USA). Briefly, 1 ug RNA was reverse transcribed using Revert Aid M-MuLV reverse transcriptase (200 U/uL), RiboLock Rnase Inhibitor (20 U/uL), 10 mM dNTP mix (1 uL) oligo dT primers in 5X reaction buffer. The cDNA was stored at -20°C for further use.

Primer Designing and Amplification

The primers for the buffalo *CSN1S1* (α-S1-casein) gene were designed using Primer-3-plus software based on the conserved sequence obtained from multiple sequence alignment analysis. These primers (*CSN1S1* forward: 5′ CTTCCCAGTCTTGGGTTCAA 3′ and *CSN1S1* reverse: 5′ ACAGCAGTTGAAGCCTTTGG 3′) are targeted to compete ORF region of *CSN1S1* gene. The Buffalo *CSN1S1* gene was amplified using gene-specific primers in Gene Pro Thermal Cycler TC-E-96G (BIOER). The PCR condition contains the following steps: initial denaturation for 3 min at 94°C followed by 35 cycles of 45 sec at 94°C, 45 sec at 58°C, 1 min at 72°C, and final extension for 7 min at 72°C. The reaction mixture (25 µL) contained 100 ng genomic DNA, 1 × Taq reaction buffer, 1 mM dNTPs, 50 units of Taq DNA polymerase, and 100 ng of gene-specific forward and reversed primer.

Cloning and Sequencing

The purified PCR products were cloned into the pJET1.2 cloning vector (Thermo Scientific, K1232). At this step, the PCR products with 3°- dA overhangs are blunted with a thermostable DNA blunting enzyme and then ligated to the linearized pJET1.2 cloning vector. The cloned PCR products were transformed into Top10 (*Escherichia coli)* competent cells. This vector contains a lethal restriction enzyme gene disrupted by ligating a DNA insert into the cloning site. As a result, only bacterial cells with recombinant plasmids can form colonies. The transformed recombinant colony was then subjected to colony PCR, and the band intensities of the amplified products were checked in 1.2% agarose gel. The desired band was eluted using QIA quick Gel Extraction Kit (QIAGEN) and sent the purified DNA for sequencing (Sanger Sequencing) to Sci Genome Lab Pvt. Ltd. (Cochin- India) (Figure 1).

Sequence Analysis

The obtained buffalo *CSN1S1* (*Bu_CSN1S1*) gene sequences were submitted to NCBI, and accession number MT276582 was received. This nucleotide sequence was assessed for homology against the publicly available database in NCBI BLASTN (https: //www.ncbi.nlm.nih.gov/BLAST), and Nucleotides sequences were then aligned with α-s1-casein gene sequences of different species by using Bioedit sequence alignment Software (version 7.2.5). After sequence analysis, the buffalo *Bu_CSN1S1* gene sequences were translated into protein sequences using Sequence Manipulation Suite (www. bioinformatics.org). Phylogenetic analysis of *Bu_CSN1S1* gene was performed by using MEGAX software (version 10.1.5) to determine the evolutionary relationship between different closely related species

Evaluation of Primary Structure of Buffalo αs1-casein Protein

The physicochemical characterization of buffalo αs1-casein protein of *Bu_CSN1S1* gene was analyzed by using ExPASy - ProtParam tool (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.

Secondary Structure Prediction

The Secondary Structure of buffalo αs1-casein protein sequence of *Bu_CSN1S1* gene was examined through

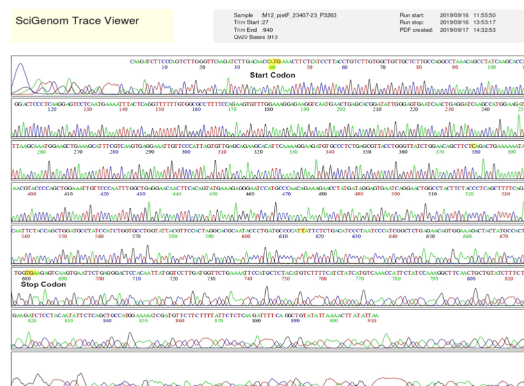

Fig.1: Graphical representation of nucleotide sequence of buffalo *CSN1S1* (alpha-S1-casein) gene

SOPMA (Self-Optimized Prediction Method with Alignment) server (Geourjon and Deleage, 1995), which computes the percentage of α-helices, β turn, and β-sheet.

Prediction of Phosphorylation and Glycosylation Sites

Different phosphorylation sites of buffalo αs1-casein protein were predicted using NetPhos 3.1 Server tool (Blom *et al.,* 1999). The glycosylation sites of αs1-casein protein were determined through NetNGlyc 1.0 Server (Blom *et al.,* 2004).

Prediction of Methylation and Acetylation Sites

The potential methylation and acetylation sites of *Bu_CSN1S1* gene-encoded αs1-casein protein were predicted by using an *in-silico* tool PLMLA (Prediction of lysine methylation and lysine acetylation) (Shi *et al.,* 2012).

Immunological Sites Prediction

The B cell linear epitopes for buffalo αs1-casein protein were predicted through IEDB analysis tool (Larsen *et al.,* 2006).

Result and Discussion

Amplification of buffalo *CSN1S1* gene and sequence analysis

The ORF region of *CSN1S1* gene of buffalo was amplified and resulting product size was 801 bp (Figure 2). The sequence analysis through the Bioedit sequence alignment program showed that there were two nucleotide changes in the coding sequence of *Bu_CSN1S1* gene at 342 bp (CTT to CTC) and 578 bp (TCA to TTA) positions (Figure 3). The translated protein sequence analysis of *Bu_CSN1S1* (αs1-casein) protein with other buffalo αs1-casein protein sequence which is obtained from NCBI database, indicated that no change at 114 amino acid position (342 bp) but 193 amino acid position (578bp) serine is replaced by Leucine (Figure 4).

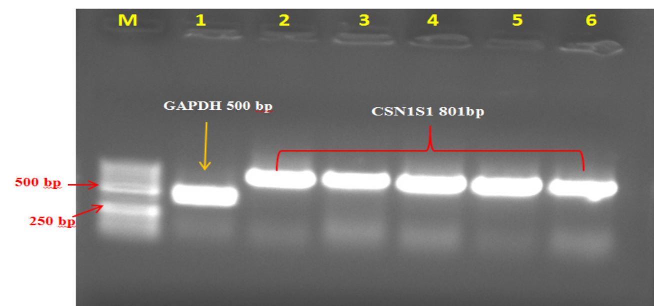

Fig. 2: Gel electrophoresis of Bu_*CSN1S1* gene PCR product using gene-specific primer. Lane – 1 GAPDH gene (500bp), Lane 2-6 *CSN1S1* gene (801 bp) PCR product, and M- 50 bp ladder

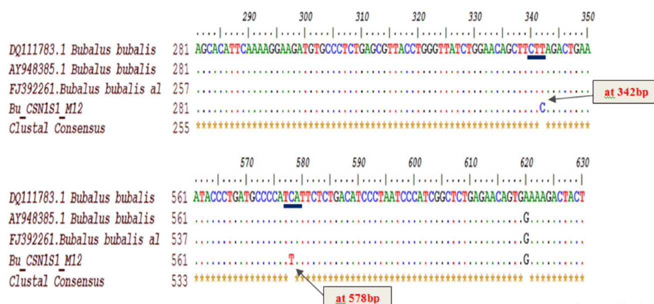

Fig. 3: Nucleotide alignment of Bu_ *CSN1S1* gene with three other buffalo *CSN1S1* genes obtained from NCBI database

Sequence Homology

The obtained nucleotide and translated amino acid sequence of *Bu_CSN1S1* gene were analyzed for the sequence homology through NCBI BLASTN and BLASTP tools, respectively. The nucleotide sequence of *Bu_CSN1S1* gene showed 99.53% identity along with 100% query cover with *Bubalus bubalis* haplotype αs1-casein (*CSN1S1*) mRNA, complete cds. The translated amino acid sequence of *Bu_CSN1S1* gene also showed a higher level of similarities with the αs1-casein isoform X2 *Bubalus bubalis* protein sequence.

Evaluation of Primary Structure

The primary structure of *Bu_CSN1S1* gene-encoded αs1-casein protein was evaluated by some parameters such as the number of amino acids, pI, molecular weight, Instability Index, Aliphatic Index, and Grand average of hydropathicity (GRAVY), represented in Table 1. The high pI value represents amino acid's essential nature, and the low pI indicates acidic amino acids. The result of ExPASy - ProtParam showed that *Bu_CSN1S1* protein is acidic. The instability index determines the stability of a protein; the value < 40 is predicted to be stable, and > 40 is unstable. In this context, our data revealed that *Bu_CSN1S1* protein is unstable as the instability index value is 56.68 (Table 1). The Aliphatic Index of a protein is 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 the aliphatic index indicates the more thermo-tolerant protein (Berjanskii and Wishart, 2008). In this study, the *Bu_CSN1S1* (buffalo αs1-casein) protein contains a high percentage of aliphatic amino acids, showing this protein's thermo-stable nature. The negative GRAVY value (Table1) for buffalo αs1-casein protein indicates the hydrophilic nature of the protein (Ertugrul and Ibrahim, 2014).

Evaluation of Secondary Structure

The secondary structure of *Bu_CSN1S1* proteins was predicted by SOPMA server. The presence of random coils defines

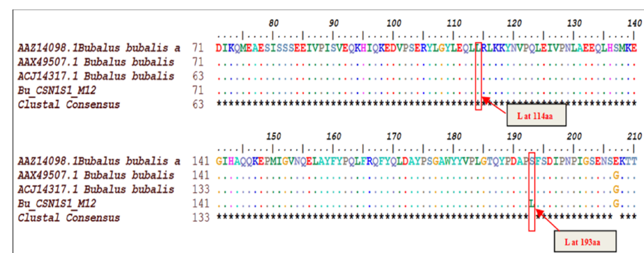

Fig. 4: proteins sequence alignment of buffalo α-s1-casein (Bu_*CSN1S1*) protein with three other buffalo α-s1-casein proteins obtained from NCBI database

protein flexibility. A high percentage (44.86%) of random coils in *Bu_CSN1S1* protein confirmed the flexible nature of the protein (Berjanskii and Wishart, 2008). Similarly, a higher percentage of alpha-helix indicated that this protein is thermo-stable since thermophilic proteins have an abundance of alpha-helices (Figure 5) (Sandeep *et al.,* 2000).

Evaluation of Phosphorylation, Glycosylation, Methylation and Acetylation Sites

Phosphorylation of protein switches the activity of a cellular protein quickly from one state to another. Thus, protein phosphorylation is a key step in various cell signaling pathways (Blom *et al.,* 1999). The insertion or deletion of the phosphate group resulted in alteration of protein function. The result of NetPhos 3.1 Server of *Bu_CSN1S1* protein (Figure 6) showed that there were 14 Serine, 6 Threonine, and 10 Tyrosine, but 11 Serine, 4 Threonine, and only 2 Tyrosine were above threshold levels, which can be predicted as phosphorylated. This result showed 17 phosphorylated residues in the buffalo αs1-casein proteins (*Bu_CSN1S1*) indicated that it is involved in signal transduction processes, cell growth, and metabolism (Batra *et al.,* 2019). The glycosylation pattern also determines the structure, folding and stability of a protein. The NetNGlyc 1.0 Server glycosylation prediction result showed that not a single amino acid residue was N-glycosylated in buffalo αs1-casein proteins, which indicated that this protein is less stable and less in the foldable state (Shental and Levy, 2008).

Methylation and acetylation sites in buffalo αs1-casein protein (*Bu_CSN1S1*) sequence were predicted through PLMLA software (Prediction of lysine methylation and lysine acetylation). It was found that there were 5 methylated lysine and 6 acetyl-lysine sites present in the buffalo αs1-casein protein sequence (Table 2). This covalent modification of specific lysine residue in this protein suggested that it plays a different role in cellular processes, including gene expression, chromosome assembly, DNA repair, etc. So the prediction of methylation and acetylation sites in buffalo αs1-casein is beneficial for identifying the protein's structural and functional properties (Batra *et al.,* 2019).

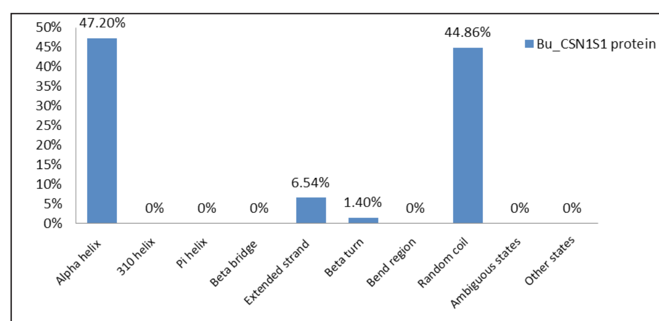

Fig. 5: Graphical representation of the secondary structure analysis using SOPMA server of Bu_*CSN1S1* protein

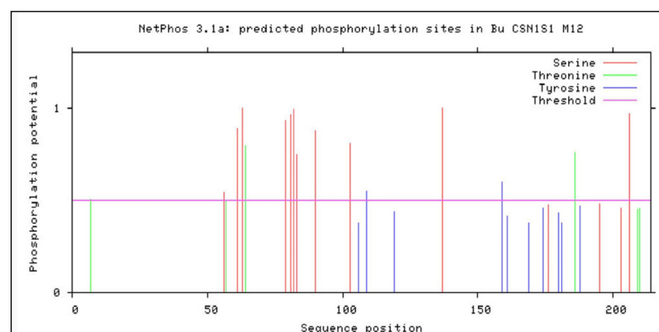

Fig. 6: Graphically representation of Phosphorylation site predicted by NetPhos 3.1 Server of Bu_*CSN1S1* protein showing different Phosphorylation sites

Prediction of Immunological Sites

Characterization of the immunological site plays an essential role in determining the antigenic nature of the protein (Larsen *et al.*, 2006). IEDB analysis result showed that in *Bu_CSN1S1* protein, there were 3 potential immunological sites for B-cell

Table 1: Physicochemical characteristics of buffalo αs 1-casein protein sequence

Physicochemical parameters Bu_CSN1S1 protein	
Number of amino acids	214
Theoretical pI	4.85
Molecular weight	24370.881
Instabiliy index	56.68
Aliphatic index	88.83
Grand average of hydropathicity (GRAVY)	-0.368
Total number of negatively charged residues (Asp + Glu)	29
Total number of positively charged residues (Arg + Lys)	18

Table 2: Different acetylation and methylation sites in buffalo αs1-casein protein (*Bu_CSN1S1*)

Position site	*Flanking residues*	*Predicted result*	*SVM probability*
22	RPKQPI-K-HQGLPQ FPEMFG-K-	acetyl-lysine	0.51882 0.7294548
49	EKVNEL	methylated lysine	0.521865
49	FPEMFG-K-EKVNEL EMFGKE-K-	acetyl-lysine	0.572703
51	VNELST	methylated lysine	0.572593
51	EMFGKE-K-VNELST PISVEQ-K-HIQKED	acetyl-lysine	0.528375
94	PISVEQ-K-HIQKED EQLLRL-K-KYNVPQ	methylated lysine	0.534337
94	QLLRLK-K-YNVPQL GIHAQQ-K-	acetyl-lysine	0.558305
117	EPMIGV	acetyl-lysine	0.580286
118	GSENSG-K-TTMPLW	acetyl-lysine	0.543894
147		methylated lysine	0.588028
208		methylated lysine	

Table 3: Different B cell immunogenic sites in buffalo αs1-casein protein

No.	*Start*	*End*	*Peptide*	*Length*
1	18	109	KQPIKHQGLPQGVLNENLLRFFVAPFPEMFGKEKVNEL	92
			STDVGSESTEDQAMEDIKQMEAESISSSEEIVPISVEQK	
			HIQKEDVPSERYLGY	59
2	113	171	LLRLKKYNVPQLEIVPNLAEEQLHSMKEGIHAQQKEP	
			MIGVNQELAYFYPQLFRQFYQL	27
3	185	211	GTQYPDAPLFSDIPNPIGSENSGKTTM	

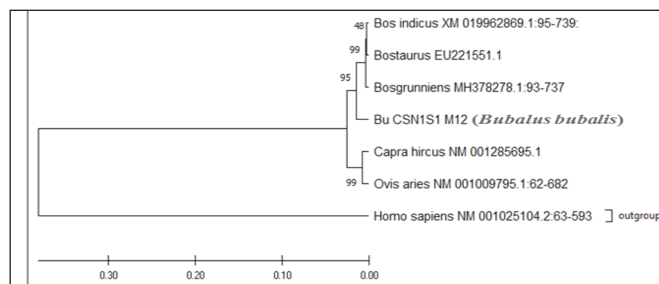

Fig. 7: Phylogenetic tree constructed from *CSN1S1* gene of different species

(Table 3), which can act as antigens. So this result indicated that buffalo α-s1-casein protein shows immunological response, and these predicted epitopic sites can be used to produce antibodies *in vivo* (Batra *et al.,* 2019).

Phylogenetic Relationship

Phylogenetic tree against buffalo αs1-casein (Bu_*CSN1S1*) gene was constructed from the nucleotide sequences of the *CSN1S1* genes of cattle (*Bos taurus and Bos indicus),* sheep (*Ovis aries*), goat (*Capra aegagrus hircus*), yak (*Bos grunniens*), and human (*Homo sapiens*) with the help of MEGAX (version 10.1.5) program (Figure 7). In the phylogenetic tree, it has been observed that cattle, yak, and buffalo formed a cluster (95%) with a closer relationship between *Bos taurus and Bos indicus (99%)*. This tree indicated that the buffalo Bu_*CSN1S1* gene is closer to the yak *CSN1S1* gene (95%). On the other hand, sheep and goats formed another cluster (99%) with a closer relationship. However, the human was placed as an out-group in the tree.

Conclusion

In this study, we found two allelic variations in the buffalo *CSN1S1* gene, which may occur due to some environmental mutation, and protein sequence analysis showed that one amino acid at 193 position changed from serine to Leucine residue. The computational analysis of buffalo α-s1-casein protein structure showed that this protein is acidic and not so stable. The hydropathicity index showed that αs1-casein was hydrophilic. It was also found that the protein has a flexible structure. Further, it was also identified that buffalo αs1-casein has more phosphorylation site but no site for glycosylation. The methylation and acetylation of a specific Lysine residue in these proteins suggested that they may be involved in DNA repair and chromosome remodeling. The presence of the different immunological sites in αs1-casein protein revealed that it acts as an antigen. The evolutionary tree concluded that the buffalo Bu_*CSN1S1* gene is closer to Yak *CSN1S1* gene.

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