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Cloning And Expression of Untagged Buffalo β-Casein in *E. Coli* and Its Purification and Partial Characterization

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Abstract:
Background*: Milk is synthesized in alveolar cells of mammary glands in response to lactogenic hormones like prolactin. It is a complex biological fluid that provides complete nutrients to neonates and comprises of proteins – caseins (80%), whey (20%), carbohydrates (lactose), vitamins, minerals and water. Milk and its products of dairy animals are also consumed by humans in later stages of life. They provide essential nutrients like proteins and also become cause of health concern like allergies in milk atopic patients. Caseins which are major allergens exist in micellar form in milk. Among other caseins, β-casein is a major milk protein that is calcium-sensitive and determines the micellar composition and structure. Phosphorylation of β casein is an essential feature and is associated with physicochemical properties and commercial features of milk. The role of β-casein phosphorylation in determining the physicochemical properties can be studied by non-phosphorylated recombinant β-casein. In this report a non-phosphorylated recombinant buffalo β casein is expressed in bacteria and further characterized.*
Materials and Methods*: Total RNA was purified from mammary epithelial cells isolated from buffalo milk. Using oligo-dT and specific primers cDNA was synthesized and the β-casein sequence was amplified and then cloned and subcloning was done for untagged β-casein expression. E. coli was transformed with the β-casein-pET 28a construct. The expressed protein was purified using precipitation and DEAE chromatography. Further β-casein was characterized by SDS PAGE and Urea PAGE at alkaline condition.*
Results*: This research reports cloning and expression of untagged buffalo β-casein in E. coli. Further an attempt was made to purify the expressed untagged β-casein using conventional methods. The untagged β-casein was insensitive to calcium and precipitation at the native isoelectric pH. The recombinant β-casein showed retarded mobility than the native β-casein.*
Conclusion: *Untagged recombinant buffalo β-casein is purified using conventional methods. The recombinant β-casein is unphosphorylated and shows no sensitivity to calcium and isoelectric pH precipitation.*
Key Word: *untagged β-casein; Buffalo; Cloning; Expression; E. coli.*

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I. Introduction

Milk, an important component of a balanced diet, is a complex biological fluid containing significant amounts of proteins, minerals, and lipids. It is recognized as a primary reservoir of top-tier proteins, offering a broad spectrum of nutritional, functional, and physiological benefits [1]. Milk is synthesized by the alveolar mammary epithelial cells of the mammary gland. During lactation, prolactin stimulates the synthesis of milk proteins including caseins in these cells [2-3]. All caseins undergo various post-translational modifications, such as phosphorylation and glycosylation, when passes through ER to the Golgi apparatus [4-5]. These modifications of caseins alter their physical properties and interacting partners [6-8]. These modifications are crucial for understanding the functional roles of caseins and decide commercial features of milk.

Lack of enough food including milk to feed human population lead to development of a concept of sustainable food. In this sequence animal free synthesis of milk by artificial casein micelle formation is being seen as an option to fulfill the requirement therefore attracted the attention of dairy industries [9-11].

Milk casein is associated with many health benefits apart from nutritional need. Caseins along with whey protein represent the predominant proteins found in milk. They exhibit properties such as antimicrobial, antioxidant, and immunomodulatory effects, among others [12-13]. On the other side caseins, including β casein, cause allergies to some milk atopic patients [14]. Milk type A1 and A2, decided by β casein sequence, is a popular issue on human health and has its commercial value.

Caseins precipitate obtained from raw, skimmed milk at 20 °C, pH 4.6 [15-16] is known as caseins fraction. This fraction comprises a heterogeneous family of protein components [17-18]. These components are separated in urea-PAGE into four types α- (αs1-, αs2-), β- and κ- casein.

Caseins fraction makes up 80% of the total protein content in bovine milk. While 95% of the casein content naturally forms casein micelles phosphate-conjugated molecules predominantly forming complexes with calcium phosphate [19-20] which are spherical colloidal particles, about 10% exists as soluble casein molecules.

Milk from different dairy animal sources like cow, buffalo etc. has specific texture and taste which are attributed majorly to properties of caseins micelles. Each casein is modified by post-translational modifications in a specific way that results of which is change in physical properties as well as interaction with other protein partners [20,8]. Role of these modifications can be studied by removing the adducts like phosphate or glycosyl substituent by chemical methods [21]. Other method to study such modifications is to prepare recombinant caseins which lacks such modifications.

Present report is part of such study which focused on cloning and expression of β casein of buffalo in E. coli. The recombinant β casein was further characterized by mobility in polyacrylamide gel electrophoresis (PAGE) and change in calcium binding property.

II. Material And Methods

Material: Bacterial strains and vectors: *E. coli* DH5α, *E. coli* Bl21pLysS, pJET1.2 cloning vector, pET28a were used in this study. Plasmid purification, gel extraction were carried out using commercial kits (Qiagen, Germany). Restriction enzymes and other molecular biology reagents were from commercial sources (New England Biolabs).

Total RNA Isolation and cDNA synthesis from buffalo milk: Total RNA was isolated by Trizol method and cDNA synthesis was done according to the manufacturer's protocol (Thermo Scientific). Synthesized cDNA was used as a template for β-casein amplification.

Primer designing and gene amplification: Based on the available predicted sequence of buffalo β-casein in the NCBI database, a set of primers pair listed below was designed:
Primers for β casein
Forward Primer-
CA***GGATCCATGG***GCAGAGAGCTGGAAGAACTC (***Bam HI*** and ***NcoI***)
Reverse Primer-
CG***CTCGAG***TCAGACAATAATAGGGAAGGGT (***XhoI***)
Restriction sites are highlighted in bold, italicized and underlined.

The coding sequence of β-casein was amplified using specific primers (mentioned above) and cDNA as a template. Amplification was carried out using Phusion master mix (Thermo Scientific) in a Peqstar 2X Thermocycler following an optimised PCR program (denaturation step at 98 for 1min, followed by 30 cycles of denaturation at 98 for 30sec, annealing at 56 for 30sec and extension at 72 for 1min with final extension at 72 for 5min) in 100µl reaction mixture. The amplified product was cloned in pJET1.2.

Cloning of β-casein in pJET1.2 cloning vector and transformation of DH5α competent cells: Competent cells of DH5α were prepared by Hanahan 1983 method. PCR product obtained from β-casein specific primers was ligated to the pJET1.2 as per the manufacturer's protocol. Thus, formed construct (β casein-pJET1.2) was used to transform the *E. coli* DH5α.

Restriction Digestion of selected colonies of β-casein: Two colonies were randomly picked from the transformed plate (β-casein-pJET1.2) and inoculated into Luria-Bertani (LB) broth containing 100µg/ml ampicillin. The next day, overnight culture was used for plasmid isolation (Miniprep Kit, Qiagen), followed by Restriction digestion using NcoI & XhoI. Clones showing correct insert release were sent for Sanger Sequencing. A clone with correct sequence, with 100% identity with available buffalo β-casein predicted sequence, was further used for subcloning.

Construction of β casein -pET28a plasmid: Plasmid, β-casein-pJET1.2, with correct sequence and empty pET28a was purified, followed by restriction digestion using NcoI & XhoI. The digested β-casein insert and digested pET28a vector were excised and gel extracted (Gel Extraction Kit, Qiagen). Double-digested and gel purified β-casein as well as pET28a were ligated using T4 DNA Ligase (New England Biolabs). The ligated product (β casein-pET28a) was used to transform *E.coli* Bl21pLysS.

Screening of expressed recombinant β-casein in *E. coli*: Transformed *E. coli* Bl21pLysS (β casein-pET28a) colonies were randomly picked and inoculated in LB medium containing 50µg/mL Kanamycin and incubated at 37 overnight in shaker incubator. Next day, this overnight culture was used to inoculate 10ml of fresh LB

medium (secondary culture) and incubated in shaker incubator at 37 until O.D reached 0.6. Then this culture was induced using 1mM IPTG (isopropyl β-D-thiogalactopyranoside) at 28 for 3hrs. Colonies of transformed *E. coli* Bl21pLysS (containing empty pET28a) were taken as a negative control and induced in the same way. After 3hours, bacterial cells were centrifuged at 3500g for 5 min at 4 , and the pellet was obtained. The obtained pellet was run on 15% SDS-PAGE to observe bands of expressed β-casein.

Purification of expressed recombinant β casein: A volume of 100ml bacterial culture was induced at 37 . After induction cells were centrifuged and the resultant pellet was used for β-casein purification. The pellet obtained from 100ml bacterial culture was lysed using 5ml lysis buffer and incubated at 4 , 30mins, followed by centrifugation at 12000rpm for 30mins. The obtained insoluble β-casein in pellet was solubilized in 4M Urea / pH 4.6 and incubated at 4 for 30mins, centrifuged at 12000rpm for 30mins. After centrifugation β-casein containing supernatant was diluted with water to bring the overall urea concentration to 1M. A solution of 1M CaCl2 was added it to obtain 100mM CaCl2 and left at 4 for 30 mins. followed by centrifugation at 12000rpm for 30mins. The obtained pellet and supernatant were then run on SDS-PAGE [22]. Recombinant β-casein containing supernatant was dialysed against 50mM Tris / pH 8.0) / 1M Urea. The dialysate was subjected to two cycles of cryo-precipitation [23]. It was done by keeping dialysate at -20°C overnight. Next day frozen supernatant was thawed and centrifuged. Obtained supernatant was repeated for overnight freeze-thaw cycle. Next day after centrifugation the obtained supernatant was subjected to DEAE chromatography.

DEAE Chromatography: DEAE Sephadex was packed up to 1mL in a 5mL syringe. The column was equilibrated with 50mM Tris / pH 8.0. The recombinant β-casein was loaded on the column. The bound protein on the column was washed with 50mM Tris pH 8.0. Then the bound protein was eluted stepwise by 50mM NaCl, 100mM NaCl and 300mM NaCl. The recombinant β-casein was analysed by SDS PAGE and Alkaline Urea PAGE [24].

III. Results

The coding sequence of β-casein gene was amplified using specific terminal primers. The coding sequence of β-casein comprises of 672 bp. A band of around same size was observed in agarose gel (Fig. 1). After PCR amplification, PCR product was used to set up ligation reaction, followed by transformation of DH5α from the ligated product. Transformed colonies were screened by colony PCR. Colonies that showed positive amplification of β-casein in colony PCR were then proceeded for plasmid isolation.

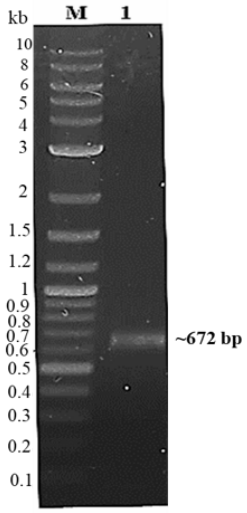

Figure 1: Agarose gel electrophoresis of PCR amplicon of β-casein using cDNA of Bubalus bubalis as template: M: Marker, Lane 1: β-casein (~ 672 bp).

Isolated plasmids of two clones were digested with restriction enzymes NcoI & XhoI. All the selected colonies showed insert release of ~ 672 bp (Fig. 2). All the constructs showing correct insert release were further confirmed by Sanger sequencing. After confirmation of 100% identity of nucleotides in the sequence, one of the constructs of β-casein was taken for subcloning. For subcloning, plasmid of one of the clones with the correct sequence, along with the pET28a vector were digested with NcoI and XhoI, then run in agarose gel further excised from the gel and purified (Fig. 3). Later these digested products were used for ligation. Ligated product, β casein-pET28a construct, was used to transform the E. coli Bl21pLysS. Positive colonies were randomly selected and induced using 1mM IPTG. Cell pellets were run on SDS-PAGE. The induced β-casein

was majorly insoluble in lysis buffer. The insoluble pellet was dissolved in 4M urea. The solubilized β-casein in 4M urea was diluted to obtain 1M urea concentration pH adjusted to 4.6. At this condition native casein precipitates but major fraction of recombinant β-casein was found in supernatant.

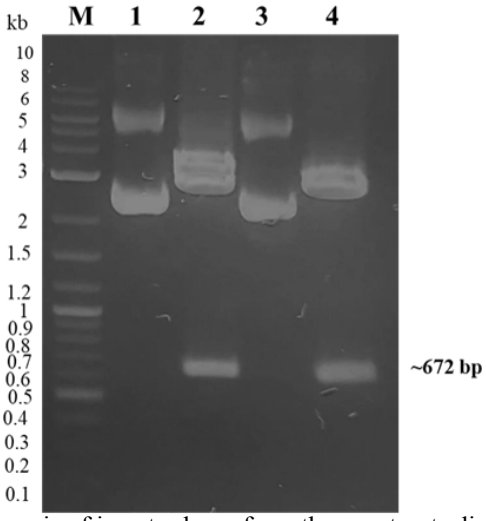

Figure 2: Agarose gel electrophoresis of insert release from the constructs digested with restriction enzymes Nco I/Xho I. M is the marker. Lane 2,4 are digested β-casein-pJET 1.2 constructs from two colonies. Lane1,3 are undigested constructs.

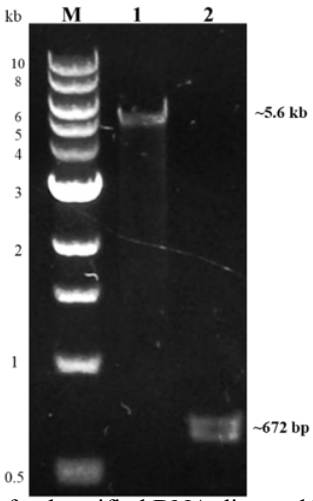

Figure 3: Agarose gel electrophoresis of gel purified DNA digested by NcoI/Xho I. Lane 1, Empty pET28a vector; lane 2, β casein-pJET 1.2 construct.

β-casein is calcium-sensitive casein therefore its solubility behavior was assessed at 100mM $CaCl_2$. For our surprise, a major amount of casein was present in the supernatant (Fig. 5). Before loading to DEAE chromatography contaminant proteins were removed by cryo-precipitation (Fig. 5). Enriched recombinant β-casein was finally subjected to DEAE chromatography (Fig 6). Purified recombinant β-casein eluted at 100mM NaCl. However, a degraded β-casein product was also observed. The purified recombinant β-casein was run in alkaline Urea PAGE. A retarded mobility of the recombinant β-casein was observed in the Urea PAGE.

IV. Discussion

Phosphorylation of calcium-sensitive caseins in milk can affect the size and composition of casein micelles and coagulation behavior on renneting [25]. Phosphate groups are covalently attached to the serine/threonine residues of caseins which can be dephosphorylated by chemical or enzymatic treatments [26-28,21]. In both cases there are chances of incomplete dephosphorylation. Chemical treatment can also modify of amino acids [21]. Recent reviews explain the need for recombinant caseins to study the role of phosphorylation in physical and sensory properties as well as commercial features like taste, texture and rheology of milk [8,29]. At current rate of production of edible protein cannot sustain the demand of projected human population by 2050 [30,10]. This compelled the search for alternatives of natural food. Animal free synthesis of milk by

artificial casein micelle formation by recombinant casein production is being seen as an option for sustained food development therefore attracted the attention of dairy industries [9-11]. Species-specific caseins can be selected to form hybrid caseins micelles to eliminate the allergic caseins from the synthesized milk. Thus, this synthesized milk would add options for milk-atopic patients [14].

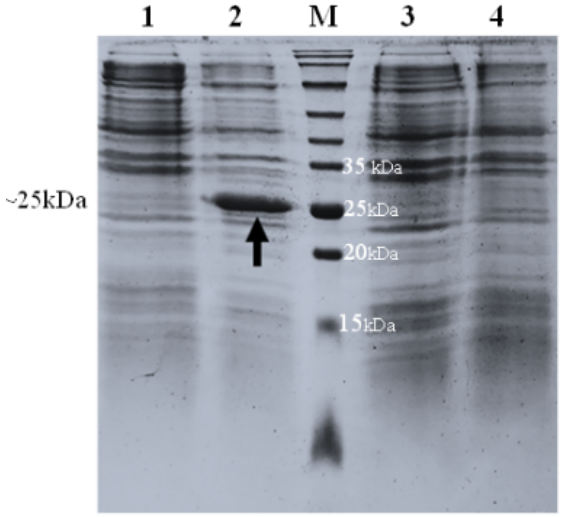

Figure 4: SDS-PAGE to check induction in β-casein-pET28a transformed *E. coli,* gel was stained with Coomassie Brilliant Blue. M is Marker; Lane 1& lane 2, β-casein-pET28a transformed clones; Lane 3 and lane 4, empty pET28a vector transformed clones; Lane 1 & 3 uninduced; Lane 2 & 4 induced by 1mM IPTG (Arrow shows β casein band of ~25kDa).

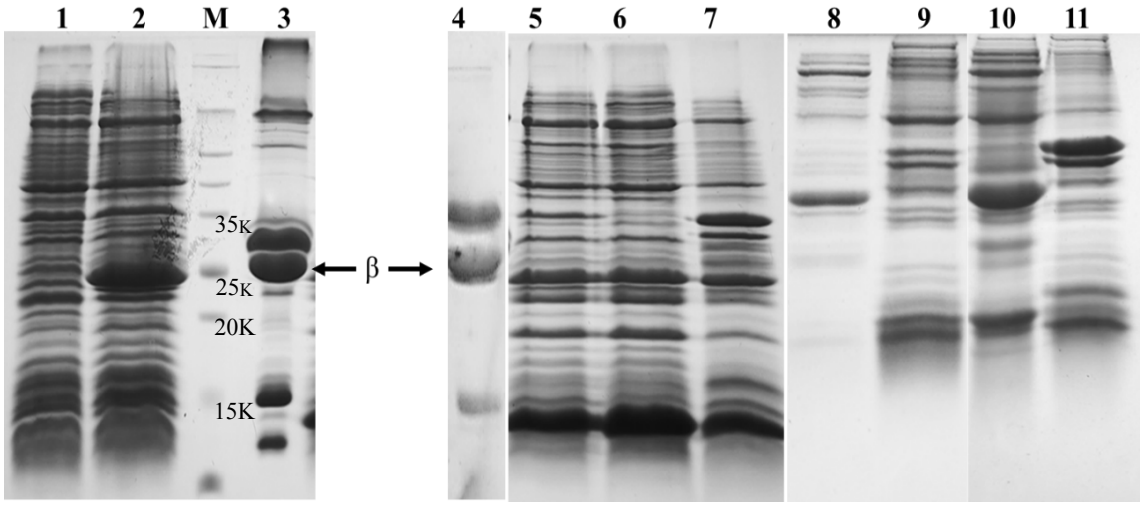

Figure 5: SDS PAGE of purification steps of recombinant β-casein. Lane 1, Uninduced; lane 2, induced; lane 3, Buffalo milk; lane 4, casein fraction of buffalo milk; lane 5, total lysate; lane 6, supernatant; lane 7, pellet; lane 8, 1M urea/100mM CaCl2 supernatant; lane 9, 1M urea/100mM CaCl2 pellet; lane 10, supernatant of cryo-precipitation; lane 11, pellet of cryo-precipitation. Arrow shows β casein in buffalo milk (lane 3) and casein fraction of buffalo milk (lane 4).

This report has shown the expression of buffalo β-casein in *E. coli*. The expressed casein showed its molecular weight of ~25kDa in SDS PAGE which was comparable with native beta casein of buffalo (Fig. 4). The expressed casein was subjected to well-known isolation and purification methods. Native beta casein precipitates at 1.7M urea and near pH 4.6 [15]. Recombinant β casein was tested for its precipitation in low urea at isoelectric pH4.6. The expressed recombinant β casein precipitation was not observed even in low concentration (1M) of urea at pH 4.6 (Fig. 5). The changed solubility at isoelectric pH indicates the absence of phosphate groups in the expressed recombinant β casein.

Native β casein is a calcium-sensitive casein which means it has very low solubility at 10mM calcium chloride concentration [31]. This property of β-casein has been attributed to the multiple phosphorylation at serine/threonine amino acid residues [26-27]. The expressed β-casein did not precipitate at 10mM $CaCl_2$ and

further it could not show precipitation even at 100mM $CaCl_2$ (Fig. 5), this further supports the absence of phosphorylation of at serine / threonine residues in the recombinant β casein.

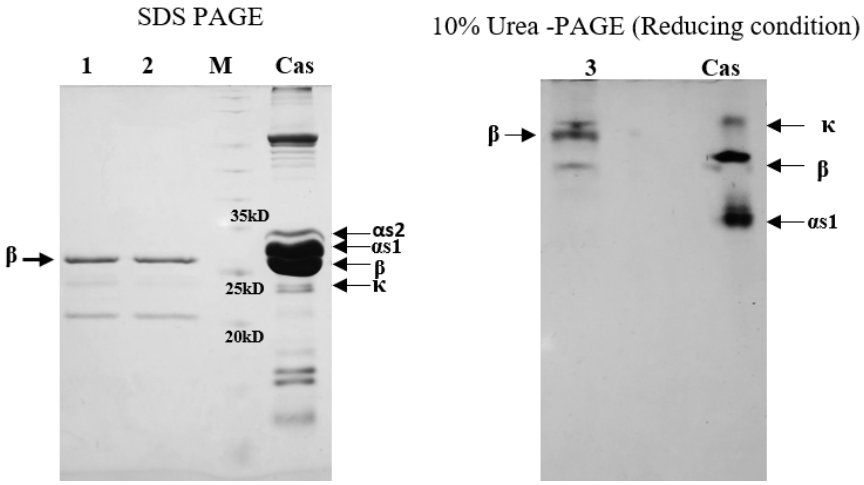

Figure 6: Purified β casein analyzed by SDS PAGE and Urea PAGE in alkaline condition. Panel: SDS PAGE Lane 1 and 2 fractions eluted at 100mM NaCl in DEAE chromatography. Panel: 10% Urea -PAGE in Reducing Condition. Lane 3, fraction eluted (same fraction as lane 1) at 100mM NaCl in DEAE chromatography. Cas is casein fraction of buffalo milk. Arrow in lane 1, 2 and 3 shows intact recombinant β-casein of ~25 kDa. Arrows in lane Cas indicate α-, β- and κ- caseins.

Soluble recombinant β-casein at 100mM $CaCl_2$ was further enriched by removing contaminant proteins using cryo-precipitation [23]. The cryo-precipitation has been used for purification of expressed recombinant proteins in E. coli. The cryo-precipitation removed few major contaminating proteins from the soluble recombinant β-casein fraction (Fig 5). Thus, the obtained soluble recombinant β-casein was subjected to DEAE chromatography. The recombinant β-casein was eluted at 100mM NaCl concentration (Fig.6).

A standard method to identify α-, β- and κ-caseins in casein fraction of milk is their mobility in urea PAGE under alkaline condition [17,32]. In the urea PAGE the α-casein moves fast and κ-casein shows the slowest mobility while β-casein has intermediate mobility. The recombinant β-casein when compared with native β-casein, as expected in the absence of phosphorylation it showed slower mobility than native beta casein (Fig. 6). This confirms that the expressed β-casein in *E. coli* has less negative charge than the native β-casein of buffalo milk. The retarded mobility is a clear indication of the absence of negatively charged phosphates from the casein molecules. Similar changes in mobility of dephosphorylated casein in urea PAGE have been reported earlier [26,28,33].

To express with an N- or C-terminal tag, like -$(His)_6$, makes a recombinant protein purification easier. A $(His)_6$ tagged casein has been expressed in *E. coli* and purified by metal ion chromatography [34]. These preparations of recombinant casein can mimic the non-phosphorylated casein. But the presence of an extra metal ion binding site, $(His)_6$ in purified casein can interfere with calcium binding. There are reports of expression of untagged casein in *E. coli* where attempts are made to purify it using advance techniques [23.35]. Present report is a protocol of purification of untagged β casein expressed in *E. coli*. The protocol applies well-known and easily available conventional precipitation and chromatography methods. At present a semi-pure non-phosphorylated and untagged recombinant β-casein of buffalo has been successfully prepared.

V. Conclusion

Recombinant buffalo β-casein has been successfully expressed in E. coli. Isoelectric pH precipitation, Calcium binding and electrophoretic mobility in alkaline urea PAGE show absence of phosphate groups in the recombinant buffalo β-casein. A purification protocol for untagged recombinant buffalo β-casein has been developed. This preparation can be utilized to understand the organization of buffalo caseins micelles. Further role of phosphorylation in determining commercial features of milk.

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