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ISOLATION AND PURIFICATION OF LACTOFERRIN FROM COLOSTRUM OF MALABARI GOATS

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ABSTRACT

Lactoferrin, an iron-binding glycoprotein, primarily present in milk, has been shown to be involved in various physiological and protective function including roles in iron homeostasis, cell proliferation, antibacterial, antifungal, antiviral, antioxidant, immunomodulatory and anticancer activities. The present study focused on the isolation and purification of lactoferrin of Malabari goats, an indigenous goat breed of Kerala. Lactoferrin is isolated from colostrum samples by cation exchange chromatography using CM Saphadex C-50 column. The isolated protein was identified and confirmed by SDS-PAGE in terms of its molecular weight. A single 80 kDa Coomassie Brilliant Blue-stained band observed in the electrophoretic profile ascertained the purity of the protein isolated. The results of the study point to a simple one step method to obtain pure lactoferrin from goats.

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1 Introduction

Lactoferrin also known as lactotransferrin, a member of transferrin family is capable of binding and transferring Fe^{3+} ions (Metz-Boutique et al., 1984). It is a glycoprotein with a molecular mass of around 80kDa and occurs as a single polypeptide chain of about 690 aminoacids. Concentration of lactoferrin is more in colostrum than in milk and it increases towards the end of lactation (Hennart et al., 1991). Other than milk, lactoferrin is also present in mucosal secretions such as tears, saliva, bile, pancreatic juice, uterine fluid, vaginal secretions, seminal fluid, small intestine secretions, nasal and bronchial secretions, urine and in specific granules of neutrophils (Masson et al., 1966). In blood plasma, the main source of lactoferrin was neutrophil degradation (Iyer & Lonnerdal, 1993). Lactoferrin possesses various biological functions including roles in iron metabolism, cell proliferation and differentiation, and antibacterial, antiviral, and antiparasitic activities. Many of these functions do not appear to be related to its iron binding ability (Korhonen & Pihlanto, 2006; Adlerova et al., 2008).

Lactoferrin exists as three different isoforms - α, β and γ. Among these,α-lactoferrin is the iron binding form. The β and γ forms have ribonuclease activity but no iron binding capacity (Furmanski et al., 1989). In terms of iron saturation, lactoferrin exists as iron-rich hololactoferrin and iron-free apolactoferrin. The C-lobe of apolactoferrin has the “closed” conformation while its N-lobe has open conformation; both lobes are closed in hololactoferrin (Jameson et al.,1998).

Lactoferrin was isolated for the first time from bovine milk (Sorensen & Sorensen, 1939) and later from human milk(Groves, 1960). Most of the pioneer research was done with respect to human lactoferrin followed by bovine lactoferrin. Human milk is the richest source of lactoferrin identified so far. Data regarding the levels of lactoferrin in the milk of other species are scanty (Adlerova et al., 2008). Goat milk is a rich source of nutrients and has its unique physical, chemical, biochemical and nutritional qualities compared to milk of other species (Slacanac et al., 2010). In addition, goat milk oligosaccharides and lactoferrin glycosylation pattern have been found to be very much similar to that of humans (Le Parc et al., 2014).

Kerala, the southern state of India, is the abode of two promising goat breeds viz., Malabari and Attappady Black, with high genetic diversity and adaptability to humid tropical stressors (Radhika et al., 2015). The genetic potential of these goat breeds and the properties of their milk have not been unraveled fully. The capabilities of the multifaceted lactoferrin protein from these indigenous goat breeds are not analysed yet. Hence the study was designed to isolate and purify lactoferrin from the colostrum of Malabari goats to harness its varied potentials.

2 Materials and Methods

2.1 Processing of samples

Around 1000 ml colostrum samples were obtained from animals belonging to Malabari goat breed maintained at University Goat and Sheep Farm, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India. The collected samples were centrifuged for 10,000 × g at 4°C for 30 minutes to remove the creamy layer and the skimmed colostrum obtained was filtered to remove traces of cream, if any. Acid whey was then prepared by adjusting the pH of diluted skimmed colostrum to 4.6 with 2N HCl. The precipitate obtained was removed by centrifuging at 10,000 × g for 30 min at 4°C. The supernatant (acid whey) was filtered and neutralized to pH 6.8 with 2N NaOH. The precipitate formed was removed by centrifugation at 10,000 × g for 30 min at 4°C The neutralized whey obtained was then subjected to ammonium sulphate based protein fraction fractionation by initial 0-45% and further 45-80% ammonium sulphate saturation. The precipitate formed after 45-80% ammonium sulphate saturation was collected and diluted in 10 mL of deionized water. The sample thus fractionated was then dialysed exclusively against several changes of distilled water and finally against 10mM sodium phosphate buffer with 250mM NaCl (equilibration buffer).

2.2 Cation exchange chromatography

Cation exchanger CM Sephadex C-50 (GE Healthcare) resin suspension was packed in a column of 300 x 18 mm size. The column was equilibrated and sample was loaded. The column was then washed with equilibration buffer until the absorbance at 280nm was less than 0.05. The bound protein was then displaced from the resin by stepwise elution protocol with different molarities of NaCl. For elution, 10mM sodium phosphate buffer containing 0.4 M NaCl, 0.6 M NaCl and 0.8 M NaCl were used as elution buffer I, II and III respectively. Initially, elution buffer I was passed slowly through the column. Fractions with a volume of 5ml were successively collected and the OD_{280} value of each fraction collected was measured by a UV- vis spectrophotometer. The elution was continued till the fractions showed a minimum OD of 0.03. Further elution of the bound protein was carried out with elution buffer II and III respectively in the same manner as that of buffer I. A graph of absorbance at 280 nm Vs fraction numbers was plotted.

2.3 Confirmation of molecular weight

The eluted fractions with maximum OD_{280} values were used for further confirmation of the protein by means of SDS-PAGE in a 12% gel using the method of Weber & Osborn (1969). Each of the eluted fraction with high OD_{280} value were loaded in the gel along with commercially available bovine lactoferrin (bLf)

(Sigma Aldrich) and wide range protein marker (10-180kDa) (Puregene, Genetix) in a predetermined order. The gel was initially run at a constant voltage of 8V/cm which was then switched over to 15 V/cm as the samples ran into the separating gel. The protein bands on the gel stained by Coomassie Brilliant Blue were imaged and analyzed by ChemiDoc™ MP Imaging System (Biorad, USA).

2.4 Estimation of concentration of the isolated lactoferrin

The fractions of Malabari goat colostrum confirmed as lactoferrin by SDS-PAGE were pooled together, dialysed against several changes of distilled water and subjected to lyophilization. These samples were reconstituted with minimum quantity of phosphate buffered saline (PBS) and then subjected to estimation of the protein content by Lowry's method using commercially available bovine serum albumin (BSA) as standard.

3 Results

3.1 Processing of samples

Cream was separated from the colostrum samples by centrifugation. Casein was precipitated and removed by addition of 2N HCl to diluted skimmed colostrum producing straw coloured, slightly turbid acid whey. It was further neutralized with 2N NaOH and centrifuged further to obtain straw coloured neutralized whey. Globulins and other high molecular weight proteins in the neutralized whey sample were precipitated by 0-45% saturation with ammonium sulphate and removed by centrifugation. The remaining proteins in the supernatant were fractionated by 45-80% saturation and collected by further centrifugation. The precipitate obtained was dissolved in minimum amount of deionized water and subjected to dialysis against equilibration buffer. This led to removal of salt as well as all the low molecular weight (15 kDa and below) proteins present in the sample, thereby retaining the remaining proteins for further analysis by means of ion exchange chromatography.

3.2 Ion exchange chromatography and elution

The sample obtained after dialysis was loaded on to chromatography column (300 x 18 mm) packed with CM Sephadex C-50 at a flow rate of 0.33ml/min and was eluted with a step gradient of 0.4, 0.6 and 0.8M NaCl. The Optical Density (OD) of the eluted fractions was monitored at 280nm and their elution profile (Figure1) was plotted. Among the fractions eluted with 0.6M NaCl, six fractions i.e., fraction numbers 32 – 37, showed high OD_{280} values and they formed a single peak in the elution profile.

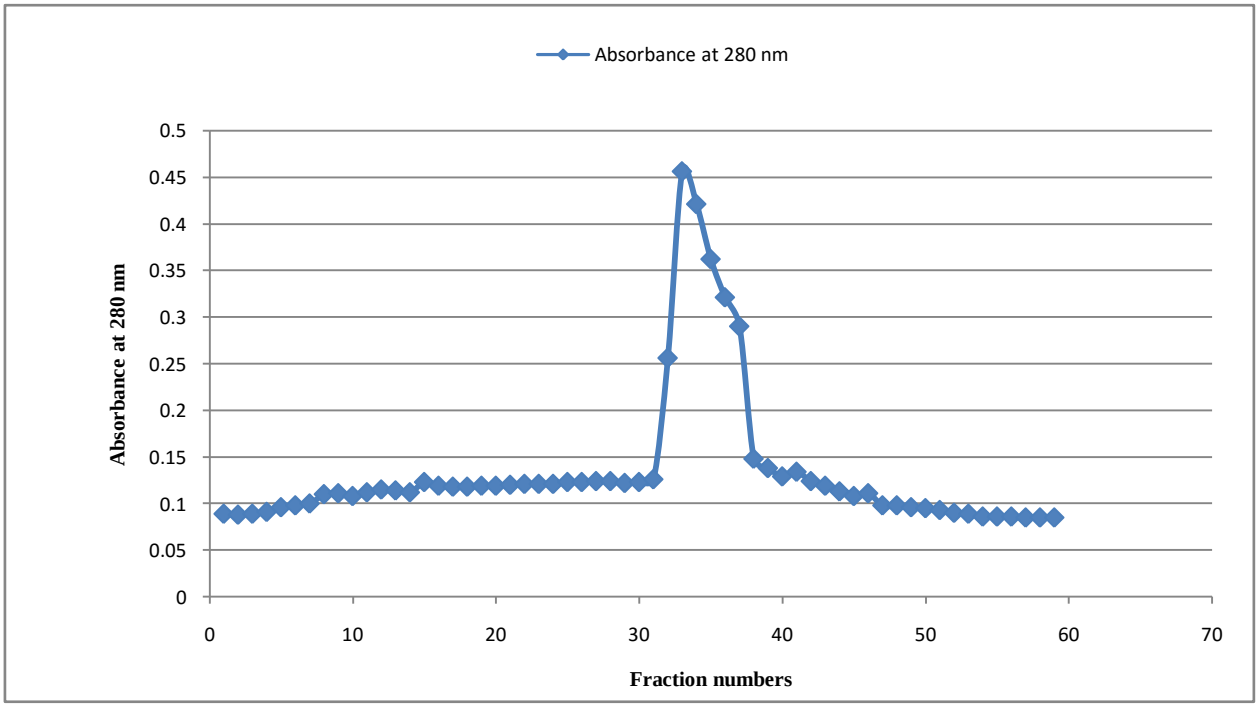

Figure1 Elution profile of fractions of Malabari goat colostrum obtained after CM-Sephadex C-50 cation exchange column chromatography (Fractions 1-25 eluted at 0.4M NaCl; fractions 25-45 eluted at 0.6M NaCl; fractions 46-59 eluted at 0.8M NaCl)

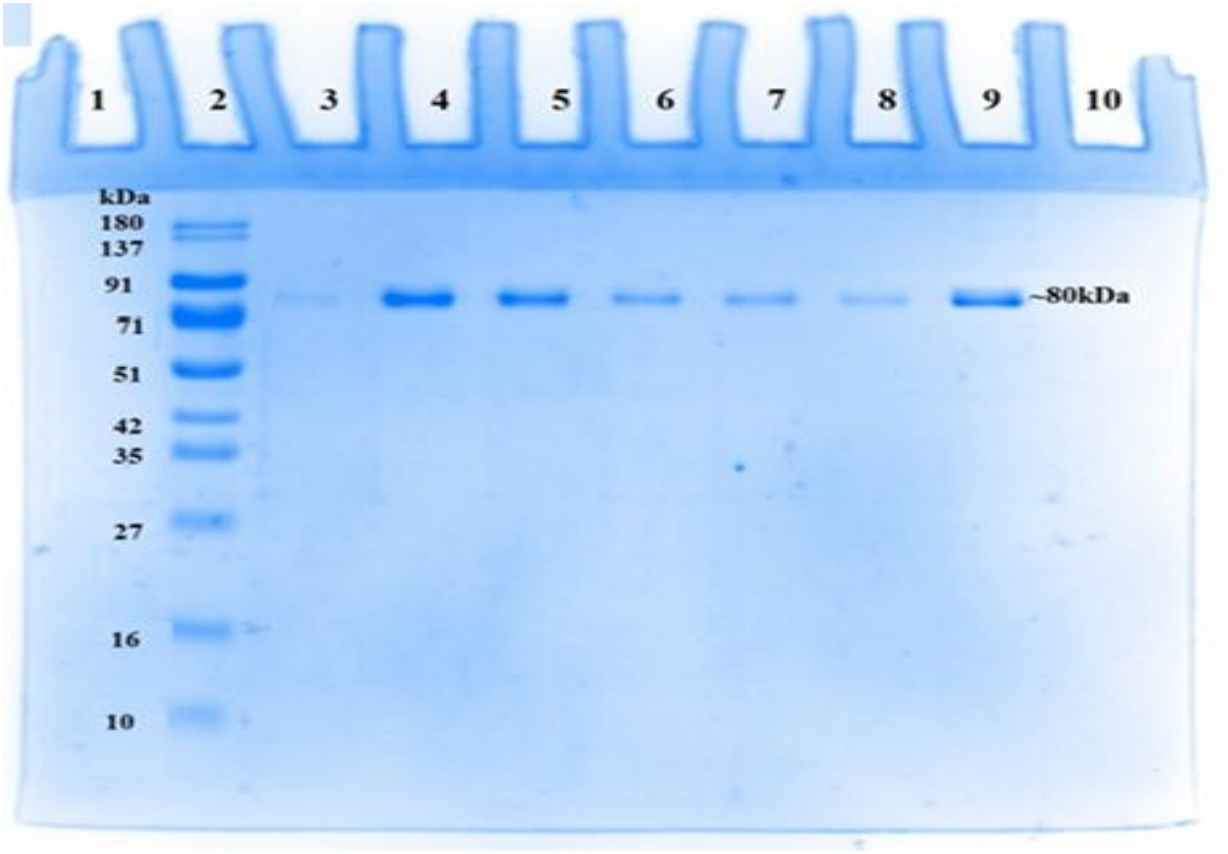

Figure 2 Electrophoretic profile of the eluted fractions with peak OD_{280} values (Lane 2: Wide range molecular weight marker; Lane 3-8: Fraction numbers 33-38; Lane 9**:** Commercially available bovine lactoferrin)

3.3 Confirmation of molecular weight

The presence of lactoferrin in the eluted fractions with high OD_{280} values was confirmed by performing SDS-PAGE followed by staining with Coomassie Brilliant Blue and the destaining thereafter. All the six fractions, i.e. fraction numbers 32 – 37, depicted a single band (Figure 2) in the gel at the same position as that of bLf, the molecular weight of which was assessed as approximately 80kDa. This single band obtained confirmed the purity and identity of lactoferrin.

3.4 Estimation of concentration of the isolated lactoferrin

A pale red coloured powder, characteristic of lactoferrin protein due to its iron content, was obtained upon lyophilization of the pooled eluted fractions having high OD_{280} values. The protein content in the sample was estimated using Lowry's method and the concentration as estimated from the standard curve was found to be 1.8 mg /mL of reconstituted sample. It corresponded to a yield of 10.94 mg/L of colostrum.

4 Discussion

Out of the indigenous breeds of goats in the country, Kerala has its own autochthonous breeds like the Malabari and Attappady Black. Functional studies on lactoferrin of such indigenous goats have not been reported yet. The present study is focused on the isolation and purification of lactoferrin from the colostrum of Malabari goats of Kerala using the method of cation exchange chromatography so that its unique potentials could be analyzed and exploited.

The colostrum samples collected from Malabari goats were effectively processed to remove casein and the whey sample was fractionated to separate globulins and other high molecular weight proteins present in it. The remaining fraction containing albumin and other minor whey proteins including lactoferrin was dialysed and subjected to cation exchange chromatography. Step wise elution protocol with different molarities of NaCl was followed; out of which 0.6M NaCl could successfully isolate lactoferrin present in the sample. Results of this study varies from previously

reported elution of caprine lactoferrin using 0.8M NaCl (Yanghoon et al., 2009) and that of bovine lactoferrin between 0.4 -0.5 M NaCl (Moradian et al., 2014).

A single band of ≈80 KDa size observed in the SDS-PAGE profile of the eluted fractions could confirm the identity as well as purity of the protein isolated. The band obtained in the upper part of the gel confirmed the presence of a protein of high molecular weight. The presence of band in the eluted fractions at the same position as that of bLF confirmed the identity of the protein as lactoferrin. Absence of any other proteins in these fractions facilitated the confirmative identification of lactoferrin by SDS-PAGE. The method of SDS-PAGE was reported to confirm the molecular weight and purity of caprine lactoferrin (Younghoon et al., 2009; Le Parc et al., 2014; Abbas et al., 2015) and that of bovine lactoferrin (Adam et al., 2008;Liang et al., 2011;Yafei et al., 2011; Moradian et al., 2014). However, the yield of lactoferrin isolated by the single-step cation exchange chromatography was found to be very less i.e., 10.94mg/L of colostrum when compared to fast protein liquid chromatography which could yield 2.4mg/mL (Moradian et al., 2014) as well as combined cation exchange gel filtration protocol which could isolate 250 mg/L of colostrum (Abbas et al., 2015).

Conclusion

Lactoferrin, the iron-binding protein, found mainly in milk, is being widely investigated for its array of biological properties such as antibacterial, antifungal, antioxidant, antiviral and anticancer activities. Goat milk is well reported for its easy digestibility, immunological properties and high content of vitamins and minerals. Goat milk and human milk possess similar type of constituents including milk oligosaccharides and glycans. In the present work, the authors describe a simple one-step isolation technique for the separation of the lactoferrin from goat colostrum which could beutilized as a therapeutic agent as well as functional food ingredient beneficial to humans and animals.

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Conflict of Interest

Authors would hereby like to confirm that there is no conflict of interests that could possibly arise.

References

Abbas ZH, Doosh KS, Yaseen NY (2015) Isolation, purification and characterization of lactoferrin from goat colostrum whey. Pakistan Journal of Nutrition14**:** 517-523.

Adam V, Zitka O, Dolezal P, Zeman L, Horna A, Hubalek J, Kizek R (2008) Lactoferrin isolation using monolithic column coupled with spectrometric or micro-amperometric detector. Sensors 8**:** 464-487.

Adlerova L, Bartoskova A, Faldyna M (2008) Lactoferrin: a review. Veterinarni Medicina Czech 53: 457–468.

Furmanski P, Li ZP, Fortuna MB, Swamy CVB, Das MR (1989) Multiple molecular forms of human lactoferrin. Identification of a class of lactoferrins that possess ribonuclease activity and lack iron-binding capacity. The Journal of Experimental Medicine170: 415–429.

Groves ML (1960) The isolation of a red protein from milk. Journal of the American Chemical Society 82: 3345–3350.

Hennart PF, Brasseur DJ, Delogne-Desnoeck JB, Dramaix MM, Robyn CE (1991) Lysozyme, lactoferrin, and secretory immunoglobulin A content in breast milk: influence of duration of lactation, nutrition status, prolactin status, and parity of mother. The American Journal of Clinical Nutrition 53:32-39.

Iyer S, Lonnerdal B (1993) Lactoferrin, lactoferrin receptors and iron metabolism. European Journal of Clinical Nutrition 47: 232–241.

Jameson GB, Anderson BF, Norris GE, Thomas, Baker EN (1998) Structure of human apolactoferrin at 2.0 Å resolution. Refinement and analysis of ligand-induced conformational change. Acta Crystallographica Section D54:1319-1335.

Korhonen HJT, Pihlanto A (2006) Bioactive peptides: production and functionality. International Dairy Journal16: 945-960.

Le Parc A, Dallas DC, Duaut S, Leonil J, Martin P, Barile D (2014) Characterization of goat milk lactoferrin N-glycans and comparison with the N-glycomes of human and bovine milk. Electrophoresis 35: 1560–1570.

Liang Y, Wang X, Wu M, Zhu W (2011) Simultaneous isolation of lactoferrin and lactoperoxidase from bovine colostrum by SPEC 70 SLS cation exchange resin. International Journal of Environmental Research and Public Health 8**:** 3764-3776.

Masson PL, Heremans JF, Dive C (1966)An iron-binding protein common to many external secretions. Clinica Chimica Acta14:735-739.

Metz-Boutigue MH, Jolles J, Mazurier J, Schoentgen F, Legrand D, SpikG, Montreuil J, Jolles P (1984) Human lactotransferrin:amino acid sequence and structural comparisons with other transferrin. European Journal of Biochemistry 145: 659–676.

Moradian F, Sharbafi R, Rafiei A (2014) Lactoferrin - isolation, purification and antimicrobial effects. Journal of Medical and Bioengineering 3**:** 203-206.

Radhika G, Raghavan KC, Aravindhakshan TV, Thirupathy V (2015) Genetic diversity and population structure analysis of native and crossbred goat genetic groups of Kerala, India. Small Ruminant Research131: 50-57.

Slacanac V, Bozanic R, Hardi J, Rezessyne Szabo J, Lucan M, Krstanovic V (2010) Nutritional and therapeutic value of fermented caprine milk. International Journal of Dairy Technology 63: 171-189.

Sorensen M, Sorensen SPL (1939) The protein in whey. Comptes-rendus Des Travaux Du Laboratoire Carlsberg 23: 55 - 99.

Weber K, Osborn M(1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. The Journal of Biological Chemistry 244 : 4406-4412.

Yafei L, Xuewan W, Mianbin W, Wanping Z (2011) Simultaneous isolation of lactoferrin andlactoperoxidase from bovine colostrum by SPEC70 SLS cation exchange resin. International Journal of Environmental Research 8: 3764-3776.

Younghoon K, Mae J, Kyoung SH, Jee YI, Sejong O, Sae HK (2009) Anticancer activity of lactoferrin isolated from caprine colostrum on human cancer cell lines. International Journal of Dairy Technology 62**:** 277-281.