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In vitro antioxidant potential of lactoferrin isolated from colostrum of kasargod dwarf cattle

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Abstract
Lactoferrin, an 80 kDa iron-binding glycoprotein possessing antibacterial, antifungal, antiviral, antioxidant, immunomodulatory and anticancer activities is primarily present in milk as a minor whey protein. The present study was focussed on the isolation and characterization of lactoferrin from the colostrum of Kasargod Dwarf cattle, a native genetic group of cattle belonging to the southern state of Kerala, India and assessment of its *in vitro* antioxidant potentials. Colostrum samples collected were subjected to CM-Sephadex C-50 cation exchange chromatography. The eluted fractions having high OD_{280} values were confirmed as lactoferrin by means of 12 percent SDS-PAGE. The total iron content of Kasargod Dwarf lactoferrin was revealed to be 600 ppm. The isolated lactoferrin was then subjected to reducing power assay and antioxidant assay. It was revealed that Kasargod Dwarf lactoferrin possesses more effective reducing power and antioxidant potential when compared to commercially available bovine lactoferrin as well as ascorbic acid, a known antioxidant.

Keywords: Lactoferrin, cation exchange chromatography, antioxidant activity, Kasargod Dwarf

Introduction
Lactoferrin (Lf) is a monomeric, non-heme, multifunctional, iron binding glycoprotein of size around 80kDa belonging to transferrin family. It is the most important bioactive component in colostrum and has higher concentration in colostrum than in milk. It is also present in secondary granules of neutrophils and exocrine secretions such as saliva, tears, nasal mucus, uterine secretions, cervical mucus and urine [1]. The isoelectric point (pI) of Lf has been calculated to be approximately 9.7.

Lf plays an important role in iron uptake in the intestine, the activation of phagocytes and mounting immune responses against various pathogens. It is capable of retarding or inhibiting the growth of many microorganisms by means of scavenging free iron and reducing its concentration in the environment of microbes [2, 3, 4]. The other functions of Lf include the regulation of myelopoiesis, hyposideremia during inflammation, iron deposition in the monocyte-macrophage system, hydroxyl radical production by neutrophils and other inflammatory as well as immunologic functions. It may be involved in establishing the neonate intestinal microflora. Lf present in mucosal secretions and neutrophil granules is considered to be part of the nonspecific disease resistance system of epithelial tissue especially the mammary gland. The antioxidant property of this protein is believed to be responsible for chemoprevention of carcinogenesis by modulating multiple molecular targets [5].

Out of the indigenous cattle breeds of India, Kerala has its own unique breed like Vechur and genetic group like Kasargod Dwarf. The present study was aimed at the isolation and purification of Lf from colostrum of Kasargod Dwarf cattle as well as the analysis of its *in vitro* antioxidant potential.

Materials and Methods
Colostrum samples were obtained from newly calved Kasargod Dwarf cattle maintained at Vechur Conservation and Breeding Unit, Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. The samples processed were initially centrifuged for 10,000 × *g* at 4°C for 30 min and the creamy layer formed on the top was removed [6]. The skimmed colostrum was filtered to remove traces of cream if any, and diluted twice with deionized water. Acid whey was prepared by acidifying diluted skimmed colostrum to pH 4.6 with 2*N* HCl.

The precipitate formed was removed by centrifugation at 10,000 × *g* for 30 min at 4°C. The supernatant (acid whey) was filtered and neutralised to pH 6.8 with 2*N* NaOH. This was again centrifuged at 10,000 × *g* for 30 min at 4°C and the precipitate was discarded. The supernatant (neutralized whey) obtained was subjected to ammonium sulphate based protein fractionation. The precipitate formed was collected, diluted in 10mL of deionized water, and was then dialysed exclusively against several changes of distilled water and finally against 10*mM* sodium phosphate buffer with 250*mM* NaCl (equilibration buffer).

The dialysed fraction was then subjected to CM Sephadex C-50 based cation exchange chromatography. The bound protein on the stationary phase was displaced by stepwise elution with 10*mM* sodium phosphate buffer containing 0.4*M* NaCl (elution buffer I), 0.6*M* NaCl (elution buffer II) and 0.8*M* NaCl (elution buffer III), respectively. The eluted samples with high OD_{280} values were used as samples for the proceeding protocols.

The obtained fractions with peak values at 280nm were analysed using 12 percent SDS-PAGE to determine their protein components and molecular weight in comparison with standard protein ie, commercially available bovine lactoferrin (bLf) (Sigma Aldrich). The gel after staining with Coomassie Brilliant Blue and consequent destaining was viewed and photographed in ChemiDoc™ MP Imaging System (Biorad, USA).

The total iron content of the isolated protein confirmed as Kasargod Dwarf lactoferrin (kdLf) was estimated according to [7] using atomic absorption spectrophotometry. Total iron concentration in the protein was calculated as follows:

$$\text{Total iron concentration (ppm)} = (AAs - AAb) \times \frac{\text{Final sample volume}}{\text{Sample weight (g)}}$$

Where,
AAs - Atomic absorption for sample
AAb - Atomic absorption for blank

The antioxidant activity of kdLf was evaluated by reducing power assay described by [8] with minor modifications. It determines the reduction potential based on the conversion of ferric to ferrous ions. Various concentrations of kdLf, bLf, and the standard antioxidant ascorbic acid (concentrations of 2-10µg) each were mixed in 1ml methanol that served as test samples. From each test sample, a volume of 0.5 ml was mixed with 0.5ml of 200mM sodium phosphate buffer (pH 6.6) and 0.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°c for 20 min. After 0.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 3000 rpm for 10 min. The upper layer (1.0 ml) was mixed with 1.0 ml of ferric chloride (0.1%, w/v) and the absorbance was measured spectrophotometrically at 700 nm. Higher absorbance indicates higher reducing power. The results were analysed statistically using simple linear regression test.

The antioxidant potential of kdLf was determined according to modified method of [9] wherein 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) [ABTS] was dissolved in water to 7 mM concentration. The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use (the radical was stable in this form for more than two days when stored in the dark at room temperature). Working reagent was prepared by diluting the stock solution with ethanol until an absorbance of 0.700 ± 0.020 at 734 nm was obtained. Thirty µL each of kdLf/ bLf/ ascorbic acid diluted with PBS (concentrations of 2, 4, 6, 8, 10, 12 and 14µg) was added to 3ml of ABTS reagent, mixed well and kept for 1 min in dark at room temperature. Absorbance was measured at 734nm. Distilled water with ABTS was kept as negative control, ascorbic acid with ABTS served as positive control while PBS served as blank.

The antioxidant activity of the sample was calculated by determining the % inhibition by using the following equation:

$$E = (A_c - A_t)/A_c) \times 100$$

Where,
A_c: absorbance of control
A_t: absorbance of test samples

The results were analyzed statistically using simple linear regression test.

Results and Discussion

Skimmed colostrum was prepared from the pooled samples collected from Kasargod Dwarf cattle. Casein in the sample was precipitated by 2N HCl to obtain acid whey. It was then neutralized by the addition of 2N NaOH and the straw coloured neutralized whey was dialysed to remove proteins below 15kDa.

The sample after dialysis was subjected to protein fractionation by ammonium sulphate. Fractionation with 0-45 percent ammonium sulphate removed globulins from the sample. The remaining proteins were fractionated and separated with 45-80 per cent ammonium sulphate. These precipitated proteins were further dialysed and subjected to cation exchange chromatography. The protocol used for processing of bovine colostrum as per [10] was followed with slight modifications.

A similar processing protocol has also been reported for isolation of goat colostrum Lf [6, 11].

The sample loaded on to CM-Sephadex C-50 cation exchange column and was eluted with a step gradient of 0.4, 0.6 and 0.8 M NaCl. All the eluted fractions were simultaneously subjected to spectrophotometric analysis to measure their absorbance at 280nm.

A single peak formed of six high OD_{280} fractions corresponding to fraction numbers 30-35 at 0.6 M NaCl could be observed in the elution profile of Kasargod Dwarf colostrum. By means of CM-Toyopearl cation exchange chromatography, Lf was separated from bovine colostrum as Lf-a which was eluted with 0.38M NaCl and Lf-b which got eluted with 0.43M NaCl [10]. A sequential chromatography by CM-Sephadex C-50-120 ion exchange column followed by affinity chromatography was reported to isolate caprine Lf [12] which was eluted by 0.8M NaCl. The same process of cation exchange chromatography using CM-Sephadex C-50 [13] was used to purify bLf in which they obtained a strong peak at a concentration between 0.4 and 0.5 M NaCl that indicated the presence of Lf.

The six eluted fractions with peak values at 280nm were analysed using 12 percent SDS-PAGE to identify their protein components in comparison with standard protein (bLf). The protein fractions eluted with the 0.6M NaCl buffer could be visualised as a single 80 kDa Coomassie blue-stained band (Fig 1).

The presence as well as purity of kdLf could hence be confirmed from SDS-PAGE since a strong, single band of protein was obtained in the electrophoretogram. The results obtained in our study are similar and compatible with other studies. The method of SDS-PAGE was reported to confirm the molecular weight and purity of caprine Lf [10, 11, 14].

The technique of SDS–PAGE was employed by [15] to determine the molecular weight of bLf which was detected as 77 kDa protein. The purity of bLf isolated was confirmed from the presence of a single band in the SDS-PAGE gel [13, 16].

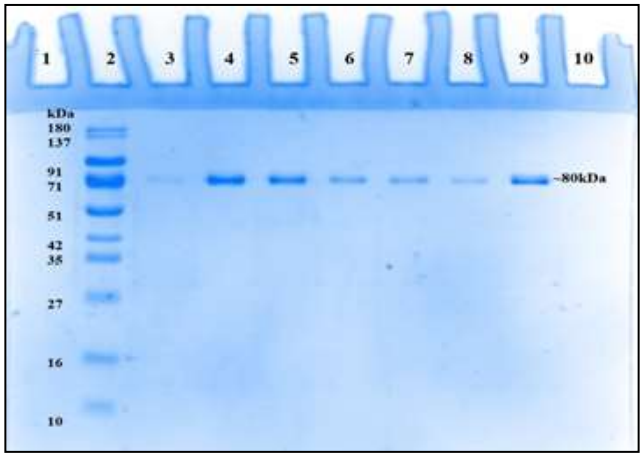

Fig 1: Electrophoretic profile of the eluted fractions with peak values Lane 2: Wide range molecular weight marker, lanes 3-8: high OD_{280} fractions, lane 9: commercially available bLf

The iron content of the isolated kdLf was found to be 600 ppm using atomic absorption spectrophotometry. The total iron content of goat colostrum Lf was reported to be 123 ppm [11].

Reducing power of the isolated kdLf was determined based on the conversion of ferric to ferrous ions indicated by a colour change of sample solution to blue. The reducing activity has a linear correlation with resistance to oxidation.

The reducing power of kdLf, bLf, and ascorbic acid increased gradually with increasing concentration (Fig 2) and the order of reduction potential was observed as kdLf > bLf > ascorbic acid.

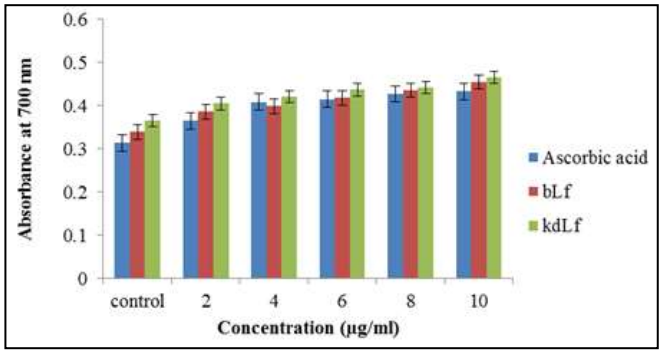

Fig 2: Reducing power of lactoferrin of Kasargod Dwarf in comparison with that of commercially available bovine lactoferrin and ascorbic acid

The concentration dependent increase in reducing power was found more in ascorbic acid compared to kdLf and bLf, using simple linear regression analysis. The order of the reduction potential was observed as kdLf > bLf > ascorbic acid. The results showed that kdLf was superior to bLf and ascorbic acid in terms of its reducing power.

Linear regression analysis showed that significant change in activity with change in concentration was more in ascorbic acid than in bLf and kdLf. The observations varied from that of [17] who stated that ascorbic acid possessed greater reducing power than bLf.

Antioxidant potential of the protein was determined using ABTS assay. The ABTS radical cation scavenging potential of the isolated lactoferrin was found to be higher than that of bLf and ascorbic acid in the order kdLf > bLf> ascorbic acid (Fig 3).

The IC_{50} value of kdLf was calculated from the graph and was found to be lower than that of bLf and ascorbic acid (Table 1).

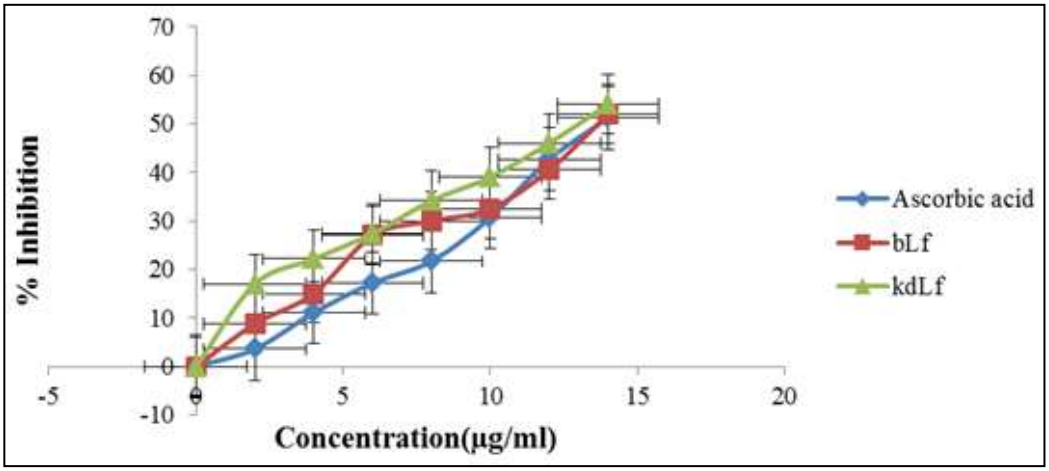

Fig 3: ABTS assay to assess the antioxidant potential of lactoferrin of Kasargod Dwarf in comparison with commercially available bovine lactoferrin and ascorbic acid

Table 1: IC_{50} values of Kasargod Dwarf lactoferrin, commercially available bovine lactoferrin and ascorbic acid against $ABTS^{\bullet+}$

Antioxidants	IC_{50} values
kdLf	12.79
bLf	13.40
Ascorbic acid	13.80

By means of simple linear regression analysis, the concentration dependent increase in antioxidant potential was found to be more in ascorbic acid compared to kdLf and bLf. The antioxidant activity of kdLf in terms of interaction with $ABTS^{\bullet+}$ was found to be in the order kdLf > bLf > ascorbic acid. The IC50 value of ascorbic acid was found to be higher

than that of bLf which in turn was found to be greater than that of kdLf. Higher the IC50 value, lower the antioxidant potential. Linear regression analysis showed that significant change in activity with change in concentration was more in ascorbic acid than in bLf and kdLf. Thus it could be observed that the antioxidant activity was highest for kdLf and that the change of variance within concentrations was more for ascorbic acid than the other two. The result contradicted that of [17] who stated that ascorbic acid possessed greater antioxidant activity when compared to bLf.

Conclusion

Lf is a multifaceted protein which is thought to play an important role in non-innate immune response, since it is primarily found in external secretions and mucosa. It has been the focus of intense research of late. Because of its immunoregulatory, antibacterial and antiviral activities, Lf is being analysed as a treatment modality for a range of infectious and inflammatory conditions in both humans and animals. The potentials of Lf from indigenous breeds and groups of livestock of India are yet to be explored. The outcome of the study promises the possibility of exploiting lactoferrin from Kasargod Dwarf cattle as an ideal antioxidant which can benefit living cells by removing and neutralizing the harmful free radicals.

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