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# Extraction and Characterization of Lactoferrin from Commercial Milk



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**DEVESH R. PARKAR, RAHUL N. JADHAV\*,  
MUKESH R. PIMPLISKAR**

*VIDYAVARDHINI'S ZOOLOGY RESEARCH  
LABORATORY, E.S.A. COLLEGE OF SCIENCE, VASAI  
(W), MAHARASHTRA, INDIA – 401202.*

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## ABSTRACT

Milk is the highest quality source of well-balanced nutrients and also displays a range of biological activities that affects digestion, metabolic responses to absorbed nutrients, growth & development of specific organs, and resistance to disease. Bioactive proteins such as lactoferrin (Lf) have been isolated over decades ago and showed their importance in stimulating the immune system in the infants through breast milk in addition to immunoglobulin present in the milk. In addition to immune system stimulation, Lf also has antibacterial activity and antioxidant activity in infant and adult of human as well as animal health. In this research paper, extraction and characterization of lactoferrin from commercially available milk has been assessed. Future perspectives of the lactoferrin have been discussed based on the results obtained.



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## INTRODUCTION

Milk, the only complete food or nutritious product, provides all the necessary nutrients to all the mammals including human from neonate age to adults due to its diverse content of nutritional compounds such as fats, carbohydrates, proteins, peptides, vitamins, growth factors, etc. Apart from nutritional compounds of milk, bioactive compounds are present in the milk in minor amounts as compared to other nutritional compounds. Research is in progress to extract these bioactive compounds on a large scale at minimum cost globally. Recent advances in research showed that neonates are protected from various microbial infections and cancer due to the presence of such bioactive compounds in the colostrum as well as the milk. As compared to the mature milk, colostrum contains higher amounts of bioactive compounds. These bioactive compounds possess multifunctional activities such as antimicrobial, anti-inflammatory, antioxidative, anticytotoxic, anticancer, immunomodulatory and mineral carrying activities. Bioactive compounds are generally in a latent state and are released upon the proteolysis of these compounds either by certain microbial enzymes released from the lactic acid bacteria which are present in the milk or during gastrointestinal or food processing (Gobbetti *et. al.*, 2002, García-Montoya,*et.al.*,2012).

Among these bioactive compounds, lactoferrin and immunoglobulin G are two important bioactive compounds in research interest which contribute to the preservation of milk itself as they possess various microbial infections and cancer-fighting properties. Lactoferrin is non-heme, an iron-binding glycoprotein with molecular weight 78 – 80 kDa that contains around 690 - 702 amino acids residues. Lactoferrin is the member of transferrin family which has a specific ability to bind iron (Legrand *et. al.*, 2008). Lactoferrin is present in mammalian secretions such as milk, tears, saliva, seminal fluids, vaginal fluids, nasal mucosa, bronchial mucosa as well as in some white blood cells and secondary granules of neutrophils (Rodrigues *et. al.*, 2009; Iigo *et. al.*, 2009). Rachman *et. al.* (2015) showed that lactoferrin concentration varies with lactation days i.e. on the 1<sup>st</sup> day of lactation it was observed that lactoferrin was more than the following lactation days. Thus, it can be seen that lactation period, age and other maternal characteristics plays an important role in the lactoferrin concentration. Lactoferrin concentration varies with breeds too.

The alteration of the activity of lactoferrin in milk could have an impact on the shelf life of raw milk and also on the development of neonates (Campanella *et. al.*, 2009). The presence of glycan molecule in the structure of lactoferrin prevents degradation of itself by proteolytic enzymes such as trypsin and trypsin-like enzymes which facilitate partial resistance to digestion in the gut. Lactoferrin is considered to be an important host defence molecule and has a diverse range of physiological activities such as antibacterial, antiprotozoal, antifungal, antiviral, anticancer, antioxidant, anti-inflammatory and immunomodulatory (Iigo *et. al.*, 2009; Parhi *et. al.*, 2012). Lactoferrin, the natural protein, is proving to be a highly promising bio drug in antibacterial therapeutic research. The use of chemotherapeutic drugs has given rise to drug-resistant bacterial infections which can be overcome by the use of lactoferrin powder or tablets as supplementary in addition to chemotherapeutic drugs at optimal concentrations.

## **MATERIALS AND METHODS**

### **Sample Collection:**

Pasteurised milk sample and tetra pack milk sample was purchased from Amul Parlour, Virar, India. All three samples were stored in a refrigerator at 4°C at Research Lab, Department of Zoology, E.S.A.College of Science, Vasai Road, until further use. All analyses were conducted in triplicate. There is no study in India about the extraction and purification of lactoferrin from bovine milk, there is a study which was done by employing Column Chromatography, Gel filtration along with its Molecular Weight and protein determination.

### **Separation of Casein:**

40mL of each sample was subjected to centrifugation for 10 minutes at 4000 rpm at 4°C [REMI CM – 8 Plus (India) centrifuge]. Fat layer (topmost) obtained was separated using a spatula and discarded. The volume of all defatted milk samples was noted and an equal volume of distilled water was added. Initial pH of each sample was recorded using the pH meter. 1N HCl (AR Grade) was added slowly with constant stirring to each sample until pH was reached to 4.6 to precipitate casein, followed by centrifugation at 2000 rpm for 10 minutes at 4°C in REMI CM – 8 Plus (India) centrifuge. Supernatants from each sample were stored in a refrigerator at 4°C for further analysis.

### **Lactoferrin Extraction:**

1N NaOH (AR Grade) was added slowly with constant stirring to all the supernatants till pH 6.0 was reached. Each sample's volume was noted and an equal volume of 45% ammonium sulphate solution was added to all samples with constant magnetic stirring at 100 rpm. Stirring was gradually increased to 420 rpm after whole addition of 45% ammonium sulphate solution and was kept for 1 hour at room temperature. All samples were then subjected to the addition of 1N HCl slowly with constant stirring till pH 4.0 was reached, followed by addition of 1N NaOH slowly till pH 8.0. At pH 8.0, an equal volume of 80% ammonium sulphate solution was added with constant magnetic stirring at 100 rpm and gradually increased to 420 rpm for 1 hour after whole addition of ammonium sulphate solution. All samples were incubated at 4°C overnight to precipitate lactoferrin, followed by centrifugation at 4000 rpm for 10 minutes at 4°C. Lactoferrin precipitate obtained was then dissolved and resuspended in 1mL 1x PBS buffer, pH 7.4 and stored in a refrigerator at 4°C in the respective tubes for further analysis.

### **Bradford's Assay:**

Bradford (1976) method was used for protein determination.

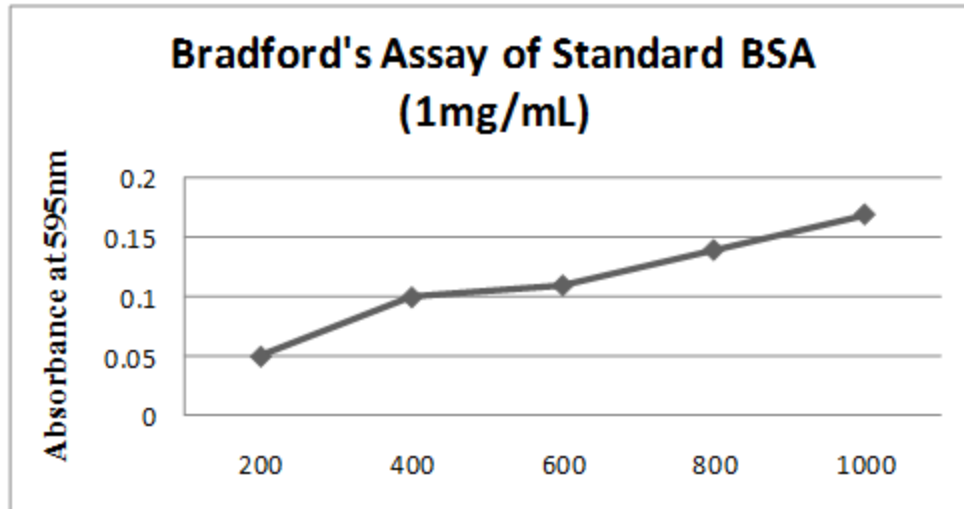
### **SDS – PAGE (Determination of molecular weight):**

Commercially available SDS-PAGE Kit was used for the analysis purpose. After electrophoresis, the gel was subjected to the 0.25% Coomassie Brilliant Blue R - 250 Staining Solution overnight at 4°C. Next day, the stained gel was subjected to the destaining solution containing methanol, deionised water and glacial acetic acid. Lactoferrin molecular weight was calculated using Rf values of Standard protein markers and extracted lactoferrin obtained by Gelanalyzer software.

## **RESULTS**

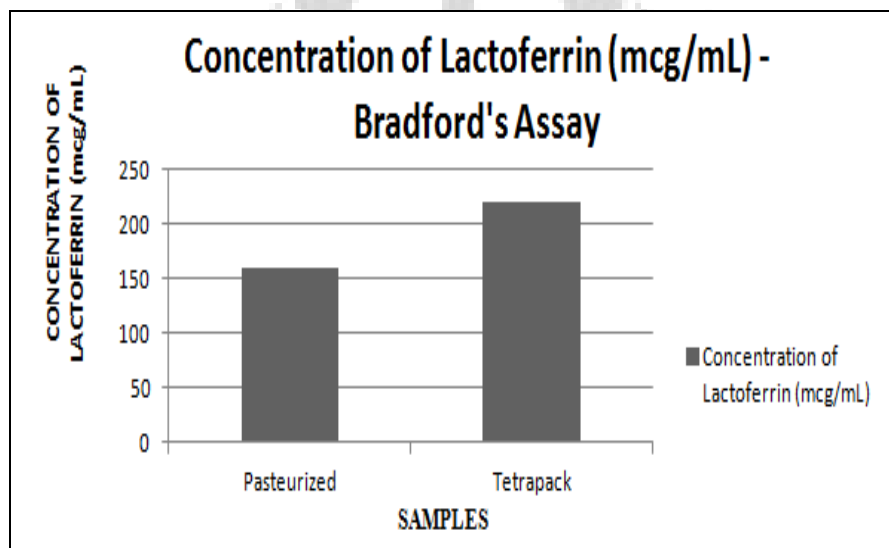
### **Bradford's Assay:**

On performing Bradford's Assay, following absorbance values at 595 nm were obtained using a spectrophotometer. Absorbance values are depicted in the following graph.



**Fig. 1. Concentration of BSA (mcg/mL)**

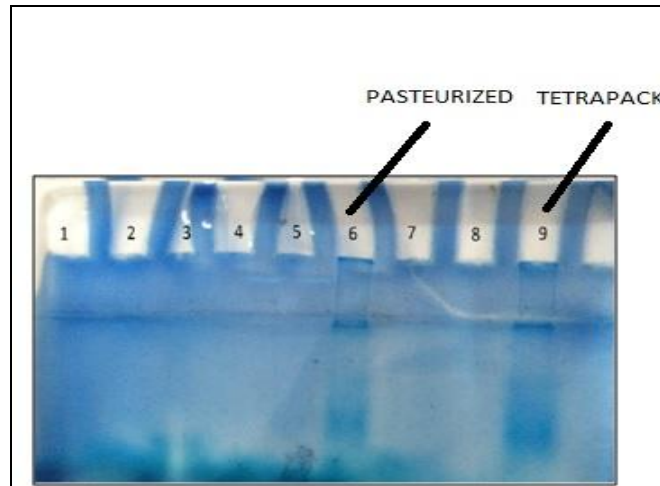
On plotting the graph of Absorbance at 595 nm against Concentration of BSA (mcg/mL), following concentrations of the unknown samples were deduced by extrapolating the standard graph.



**Fig.2. Concentration of Lactoferrin**

### SDS-PAGE Analysis:

On performing SDS-PAGE of the samples, following bands were obtained which can be seen in the following gel image.



**Fig. 3. Polyacrylamide gel showing blue stained bands of Lactoferrin in the good nos. 6 & 9.**

#### **Purity of the Lactoferrin:**

A single band of lactoferrin obtained in the good nos. 6 & 9 on performing SDS-PAGE, implies that the relevant samples contain lactoferrin in pure form.

#### **DISCUSSION**

It can be said from the results that Amul Tetra pack & Amul Pasteurised Milk contains a high concentration of the lactoferrin which can help in the eradication of many pathogenic bacteria by consuming the milk as a nutritional supplement or by consuming the purified Lactoferrin powder as an aid to stimulate the immune system and also to eradicate the bacteria. Presence or characterization of lactoferrin is confirmed by performing the SDS-PAGE. The purity of extracted lactoferrin is confirmed by SDS-PAGE since a single band of lactoferrin was obtained, it can be said that the extraction procedure gives pure lactoferrin subject to the presence of small traces of ammonium sulphate salt but it is avoidable if the lactoferrin is not intended for human consumption purpose. The results of this study similar compatible with other studies. Younhoon *et.al.* (2009) used SDS-PAGE to confirm the purity of caprine lactoferrin. Yafei *et.al.* (2011) also obtained a single band in the gel of SDS-PAGE to confirm the purity of isolated lactoferrin from defatted bovine colostrums. Further, SDS-PAGE also used to confirm lactoferrin purity which was isolated from goat colostrums whey (Zainab *et. al.*, (2015) and cow's milk by using CM –Sephadex C-50 (Moradian, 2014). Adam *et. al.* (2008) employed SDS–PAGE in determining the molecular weight of cow milk lactoferrin which was detectable AS 77 kDa.

Annabelles *et. al.*, (2014) determinate the molecular weight of goat milk lactoferrin by SDS-PAGE as 78 kDa. Liu *et. al.* (2016) using MALDI-TOF-MS, which showed the molecular weight peak at 84,100 Da (84 kDa). Thus, the molecular weight of the extracted lactoferrin from both the samples was estimated to be 80-88 kDa by comparing the gel with the standard pre-stained protein marker gel obtained from Bio-Rad Laboratories, India and from the values obtained by extrapolating the standard graph of protein molecular weight. Hence, we are in agreement with the studies conducted by above-said researchers.

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