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Isolation of Lactoferrin from Camel Milk through Fast Protein Liquid Chromatography and its Antagonistic Activity against *Escherichia coli* and *Staphylococcus aureus*

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Authors' Contribution
This paper is from the PhD research work of BN. TZ had done supervision of research work and helped in making experimental design. MAR and AJ were members of supervisory committee. MAR helped in formatting and critical revision of the paper. AJ helped in drafting the article and final approval.

Key words
Lactoferrin, Camel milk, Fast protein liquid chromatography.

ABSTRACT

Lactoferrin, a multifunctional glycoprotein, occurring as awhey constitute in milk secretions of animals and humandisplays a variety of antimicrobial, antioxidant and immunomodulatory as well as a number of other biological functions. Its potential can be exploited in several food applications. Keeping in view the increased demand of natural antimicrobial agents to control prevalence of food borne pathogens in final packaged food products as well as for food preservation/ biocontrol, this study was designed to isolate lactoferrin from milk of camel. Cation exchange resin (CM-Sephadex C-25) was used for the isolation of lactoferrin using the fast protein liquid chromatography. The lactoferrin was further characterized through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Disc diffusion method was applied for the evaluation of antibacterial activity of lactoferrin. Minimum inhibitory concentration (MIC) of lactoferrin was determined by 96-well plate method using ELISA reader. Results depicted that CM-Sephadex C-25 was a good cation exchanger for recovery of lactoferrin. Camel raw milk produced 2.4 mg/mL of lactoferrin. The lactoferrin isolated from camel milk exhibited promising antibacterial activity against *E. coli* and *Staphylococcus aureus*. Concentration of lactoferrin *i.e.* 4,000 ppm (4 mg/mL) showed the best results against both the pathogens. The *E. coli* was more susceptible to inhibition than *S. aureus*in disc diffusion assay. The MIC of lactoferrin was found to be 0.5 mg/mL for *E. coli* and 0.2 mg/mL for *S. aureus*.

INTRODUCTION

Camel milk is considered to be one of the key components of the human diet as well as that of several traditional medicines in many parts of the world. It has a high biological value due to higher contents of antimicrobial factors such as lysozyme, lactoferrin (Lf) and immunoglobulins (El-Fakharany *et al*., 2008; El-Agamy *et al*., 1992). Bioactive components in camel milk comprise Lf, lactoperoxidase, lysozyme and insulin like protein (El-Agamy, 2009). Biological roles are primarily owing to the peptides and milk proteins. Major (α-lactalbumin, β-lactoglobulin, bovine serum albumin, immunoglobulins, glycomacropeptides) and minor (lysozyme, lactoperoxidase and lactoferrin) milk proteins are the main constituents of whey proteins (Park *et al*., 2007). Anti-microbial perspective of milk is mainly accredited due to lactoperoxidase, lysozyme, The Lf and immunoglobulins that acquire imperative dietetic and nutraceutical prospective with special reference to promotion of health and impediment of diseases (Madureira *et al*., 2007). Antimicrobial factors are significantly present in higher concentrations in camel milk than those in cow or buffalo (El-Agamy, 2000). An anti-infective, Lf, is minor but mighty bioactive component of milk playing amazing role, not only in weight management but also as a natural alternative to synthetic antibiotics. Food and Drug Administration (FDA) certified the Lf as "Generally Recognized as Safe (GRAS)". The application of naturally occurring Lf to food preservation (antimicrobials) is gaining more and more attention because of consumer's trend of taking natural compounds/ food additives in their foods. Recently, Lf has attracted more attention because of the increased problems of antibiotics as antibiotic resistance, direct toxicity, hypersensitivity, antibiotic induced immunosuppression and super infections. These problems have high-lighted the need for non-antibiotic therapy by using novel immunomodulators, and Lf is one of the highly promising agent to combat this issue (El-Hafez *et al*., 2013).

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In Pakistan, malnutrition and food adulteration in addition to pathetic hygienic conditions are among the most attention grabbing areas requiring instantaneous attention. Like other underdeveloped countries, diarrhea is a real menace especially to infants and children, and in general considered as a major and challenging public health concern in the country. This alarming situation gets even bleak with the protozoan and viral intervention (Soofi *et al*., 2011). Innovative hurdle technologies are needed to resolve issues of food preservation in developing as well as developed countries. Regarding food safety situation in Pakistan, thousands of people die because of poor and substandard hygiene in food chain from farm to fork. Emerging food-borne as well as zoonotic pathogens cause several outbreaks of food borne diseases in human beings. Antibiotic-resistant bacteria are the major issue posing a serious challenge for the scientists from health sector. Similarly, because of swiftly prevailing conditions of malnutrition around the world, there is a dire need to extract out functional ingredients from the food of animal origin. Therapeutic benefits of camel lactoferrin (CLf) cover a broad range including inhibition of HCV entry and replication inside human peripheral blood and, it has proven to be effective without an adjunct therapy (Bonkovsky *et al*., 1997). In addition to inhibition of hepatic disorders, CLf helps in improving the production of antioxidant enzymes (Al-Hashem *et al*., 2009). The CLf not only inhibits lipid peroxidation but also regulates the hepatic iron content unlike other Lfs (Khan *et al*., 2001; Konishi *et al*., 2006).

MATERIAL AND METHODS

Sample preparation

The freshly procured camel milk sample from local dairy farm in Faisalabad was prepared as follows:

De-fatting (100 mL) was done by centrifugation at 2500 x g for 30 min at 4°C. Casein was removed by precipitating skimmed milk (fat removed) with 1N HCl adjusting pH to 4.6; centrifuged again for 15 min; whey was filtered through Millipore filter (0.45μm) to completely remove the casein precipitates; the filtrate (whey samples) was frozen at –20°C to avoid microbial spoilage during the experimental period and prior to proceeding for further analysis after Moradian *et al*. (2014).

For whey protein precipitation, the salting out was done after Masson and Heremans (1967). The protein was precipitated from the crude extract using $(NH_4)_2SO_4$ as described by Moradiain *et al*. (2014) where the ammonium sulfate was added upto 20% (*w/v*), while saturating at 4°C and centrifuged at 10,000 x g for 20 min; the pellet was preserved in phosphate buffer. Ammonium sulfate was added to the supernatant to achieve 30, 40, 50, 60 and 70 % (*w/v*) saturation keeping the temperature constant. All of the precipitates were collected by centrifugation at 10,000 x g for 20 min. Thereafter, it was re-suspended in 50 mM phosphate buffer (pH7.0) and dialyzed against the same buffer for 12 h.

Dialysis of the sample after ammonium sulfate precipitation was done after Masson and Heremans (1967). The dialysis tube was cut into the required length, placed in 2% sodium carbonate solution (pH 8.0), and boiled in a hot water bath for 10 min. Sodium carbonate solution was then decanted. The dialysis bag was rinsed thrice with distilled water by keeping it in boiling water bath for 10 min. The distilled water was decanted. After the third time, the dialysis bag was boiled in 10 mM EDTA (pH 8.0) in the water bath for 10 min. The dialysis bag was allowed to cool down at room temperature and stored at 4°C; water was added to the dialysis bag to check leakage. The sample obtained after ammonium sulfate-precipitation was poured into the bag and placed in a solution of 100 mM phosphate buffer (7.0) at 4°C for 12 h. The buffer was replaced after every 3 h.

Fast protein liquid chromatography (FPLC) analysis

The partially purified and dialyzed CLf fraction was serially loaded to 5 x 30 cm column packed with CM-Sephadex C-25 for cation exchange chromatography (FPLC, Bio-Rad) following Moradian *et al*. (2014) at a flow rate of 3mL/min that had been previously equilibrated with 50mM phosphate buffer (pH 6.7). Thereafter, the protein-loaded column was washed with 500 mL of the same buffer to remove loosely and unbound sample components. Bound protein was eluted with a linear gradient of 0-1 M NaCl (total volume of 200 mL) in the phosphate buffer. Fractions of 2 mL were collected at the flow rate of 4mL/min. Protein-containing fractions were checked for purity, and the active fractions were pooled, dialyzed against the same phosphate buffer and preserved (Moradian *et al*., 2014).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of CLf was checked through electrophoresis using SDS-PAGE. The molecular mass was determined by applying the purified Lf on 10% SDS-PAGE. The resolving gel mixture was poured into the gel apparatus already assembled. After pouring, *n*-butanol was layered on the top of the resolving gel to get an even surface. Then *n*-butanol was removed and top of the gel surface was washed 3-4 times with distilled water. The stacking gel mixture was then poured on top of the polymerized resolving gel. The comb was immediately inserted and

stacking gel was allowed to polymerize at refrigerated temperature. Dialyzed fractions of ammonium sulfate-precipitated whey were mixed in 2X sample loading buffer and boiled for 3 min for the binding of SDS to the protein prior to loading on the gel. Protein marker with molecular mass ranging from 10-200 kDa was also run as the standard. Protein ladder was applied directly to SDS-PAGE. The 5X running buffer was prepared already as described earlier. The SDS-PAGE was initially run at 80 volts and then brought upto 120 volts after 30 min. The PAGE was stopped when tracking dye front reached the bottom of the gel.

Bradford assay (Quantitative analysis)

Bradford assay was performed for the quantification of CLf in the samples (Abbas *et al*., 2015; Moradian *et al*., 2014; Bradford, 1976). Bovine serum albumin (BSA) was prepared for standard curve.

Antibacterial activity of CLf against E. coli *and* S. aureus

The fresh cultures of *E. coli* and *S. aureus* were obtained from the Biochemistry Lab, University of Agriculture, Faisalabad, These were maintained on tryptic soy agar (TSA) slants, sub-cultured periodically to maintain viability and stored at 4°C. Antimicrobial activity of Lf was done by disc diffusion method after Nikolic *et al*. (2004). Sterile round discs (6 mm) were soaked in 100μl of each of the Lf extracts. The pure cultures of *E. coli* and *S. aureus* were grown on Muller-Hinton agar (beef extract, 300g/L; casein hydrolysate, 17.5g/L; starch, 1.5g/L and agar, 17.0 g/L) plates and discs containing different concentrations of samples were placed in them. An antibiotic was used as standard. The plates were then incubated overnight at 37°C to check the pathogen growth.

Determination of minimum inhibitory concentration (MIC)

The MIC was determined with a slight modification in the methods described by Mishra *et al*. (2013) and Sarker *et al*. (2007). All the tests were performed in 96-well plate using Muller-Hinton broth as growth medium. A 50μL volume of the nutrient broth was poured into each well. The culture of *E. coli* O157:H7 and *S. aureus* were prepared in tryptic soy broth (TSB) and adjusted so that the final concentration came out to be $1x10^9$cfu/mL, approximately. A 10μL fraction of this culture was added to each well. In addition, 50μL of the extract (Lf) was also added to each well.

Two-fold serial dilutions were prepared for each of the extract that was added, i.e. 100, 50, 25, 6.25, 3.12, 1.56, 0.78, 0.39 % (*v/v*) and so on. A control was also prepared with an antibiotic. The plates were incubated overnight at 37°C. In order to confirm MIC, 0.1 mL of the broth was removed from each of the wells and surface plated/ spread on TSA. After aerobic incubation at 37°C, the plates were read in ELISA reader, and the numeric value for each well was recorded. From this numeric value the MIC for each extract was estimated as the lowest concentration, which resulted in a significant decrease in inoculums' viability (> 90%).

RESULTS AND DISCUSSION

The Lf isolated from camel milk whey using CM-Sephadex C-25 attached with FPLC was eluted at higher molarity. The results reflected that camel milk contained appreciable quantities of Lf (Fig. 1). The results also revealed that the maximum amount (2.3 mg/mL) of the Lf was recovered from the unpasteurized milk. Conesa *et al*. (2008) isolated Lf from milk of different species including camel using SP-Sepharosecation exchange resin. The CLf was identified as band of 80 kDa and one peak under linear gradient of 1M NaCl. While studying its thermal behavior, CLf's thermogram showed similar appearance as that of the human milk. However, the values of maximum heat absorption, onset temperature and enthalpy change of denaturation are lower for the CLf.

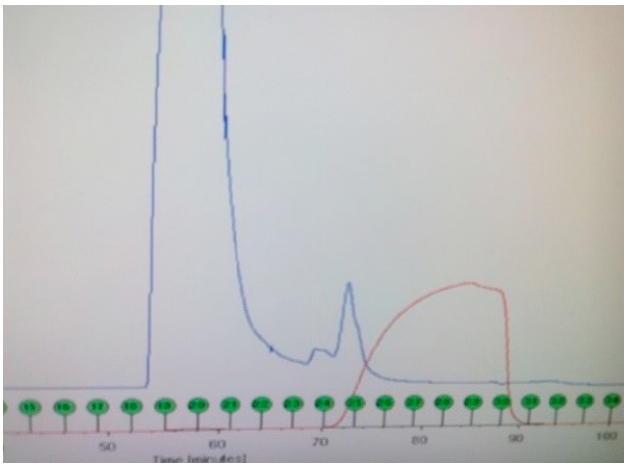

Fig. 1. Fast Protein Liquid chromatogram of Lf isolated from Camel milk.

Duhaiman (1988) suggested that CLf had molecular weight of 78 kDa. The present studies are in accordance with El-Agamy *et al*. (1992), who demonstrated variances between proteins of camel and bovine milk. Differences in composition and structure between camel and bovine milk defensive proteins lead to some of the differences in activity. About the content of Lf in camel milk, several reports have illustrated different levels of this protein. But, different workers adopted different units, methods and

approaches for quantitative analyses. The findings of the present study were quite in line with those of El-Hatmi *et al*. (2006), who had opted the same methodology as was performed in the present study. It was observed that the concentration of Lf and IgG in camel milk was somewhat higher than that appeared in the cow milk. However, only this reason does not seem justifying the medical properties accredited to camel milk. Further investigations are required to dig out the antibacterial activity of CLf in real sense and to compare it with bovine Lf .

Characteristics of SDS-PAGE

After SDS-PAGE, molecular weight of the Lf was calculated from drawing the relationship between the logarithms of the molecular weight for standard proteins compared to relative mobility. Voltage was maintained constantly till the tracking dye reached the gel bottom. Figure 2 reveals the molecular weight of CLf as 76 kDa. Migration pattern of the CLf was slightly different from those of others. It was slower than the cow and buffalo milk Lf. El-Agamy *et al*. (1996) isolated and characterized CLf by carboxymethylcellolose and SDS-PAGE, respectively, and compared it with bovine milk protective proteins, including lysozyme, lactoperoxidase and Lf. Many differences between bovine and camel milk proteins were noticed during the purification and characterization studies.

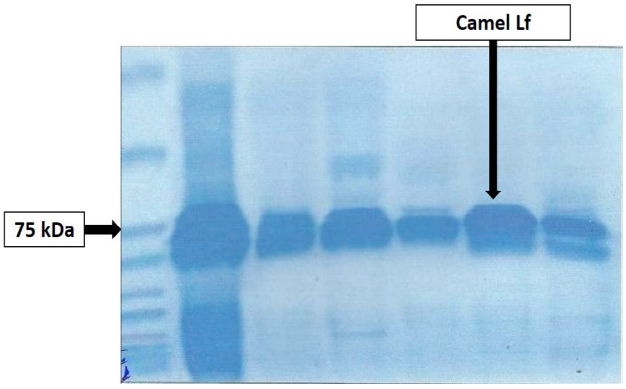

Fig. 2. SDS-PAGE of lactoferrin isolated from camel milk through fast protein liquid chromatography.

Antibacterial activity of Lf

The statistical results of the effect of Lf from camel milk on the pathogenic bacteria and their mean values have been presented in Table I. Similarly, Figure 3 depicts the difference in antibacterial activity of various concentrations of CLf on *E. coli* and *S. aureus*. Application of different concentrations of CLf revealed an increase in zones of inhibition with the application of increasing concentrations and the effect started from the lower concentration *i.e.* 50 ppm (10mm) to 4000 ppm (28mm) in case of *E. coli* and 500 ppm (10ppm) to 4000 ppm (20mm) in case of *S. aureus*. Antagonistic effect of CLf against *E. coli* and *S. aureus* was found to be the very potent.

Table I.- Mean values for antibacterial activity of different concentrations of camel Lf (CLf) against *E. coli* and *S. aureus.*

Treatments	Zone of inhibition (mm ± SD)	
	E. coli	***S. aureus***
Positive control (Antibiotic)	(Ampicillin) 22±1.0^{bc}	(Streptomycin) 25±0.578^{ab}
Negative control (Zero Lf)	NZ	NZ
Lf. Concentration (ppm)		
T1 (1)	NZ	NZ
T2 (10)	NZ	NZ
T3 (50)	10±0^{f}	NZ
T4 (100)	15±1.0^{e}	NZ
T5 (500)	21±1.732^{cd}	10±1.156^{f}
T6 (1000)	25±1.0^{ab}	15±0.578^{e}
T7 (2000)	26±1.0^{a}	18.66±0.333^{d}
T8 (4000)	28±1.732^{a}	20±0.578^{cd}

P < 0.05; ± stands for standard deviation among n = 3 experiments. Values are taken as means of three observations.

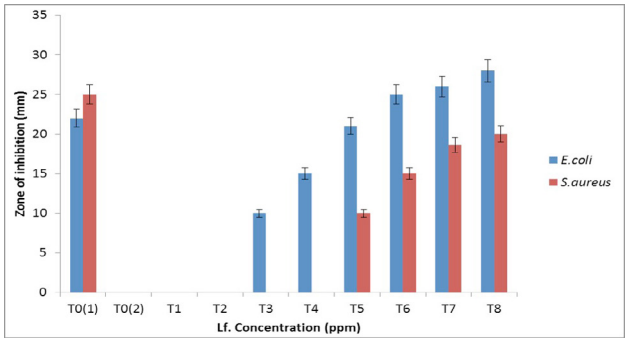

Fig. 3. Effect of different conc. of CLf against *E. coli* and *S. aureus*.T_0(1), Positive control: standard antibiotic (ampicillin and streptomycin); T1(2), Negative control (disc without Lf); T1, 1ppm; T2, 10 ppm; T3, 50 ppm; T4, 100 ppm; T5, 500 ppm; T6, 1000 ppm; T7, 2000 ppm; T8, 4000 ppm.

The values of zones of inhibition calculated for all the concentrations of Lf against *S. aureus* revealed that 4000 ppm concentration of CLf restricted the growth of pathogen upto 20 mm, although, CLf concentration ranging from 50-2000 ppm was also effective against *S. aureus*. As a whole, CLf was found to be more effective

against *E. coli* as compared to *S. aureus;* however, it also showed promising activity against *S. aureus* which was difficult to kill by other antimicrobials.

The antibacterial activity of Lf has, in fact, been a particular field of interest of a number of scientists. Conesa *et al*. (2008) studied the antibacterial activity of CLf, and demonstrated that CLf was the most potent antimicrobial protein amongst all other sources including human and alpaca. In an earlier study, Al-Majali *et al*. (2007) measured the antibacterial activity of CLf against *E. coli* and *S. aureus* isolated from the mastitic milk samples, and reported that the CLf was able to hamper the growth of *S. aureus* upto 20%, *S. agalactiae* 2% and streptococci upto 12%. A few years later, another contribution was added when El-Agamy and co-workers (El-Agamy *et al*., 1992) extracted proteins, such as Lf, lysozyme, lactoperoxidase and immunoglobulin A form the camel milk and used them to study their antibacterial activity against *Lactococcus lactis*, *E. coli*, *S. aureus*, *Salmonella typhimurium* and rotavirus. Similarly, Abdel-Salam *et al*. (2014) studied the effect of CLf against human pathogens using Delvo test method and disk diffusion assay, and reported a 2.5 mg/mL concentration of CLf effective to inhibit the activity of *S. aureus, S. epidermidis, Proteus mirabilis, E. coli* and *K. pneumoniae.* Results of the recent study are, though, quite in line with these findings, yet the author is of the view that further investigations are needed to elucidate the mechanism of resistance of some bacterial species to CLf. Yassin *et al*. (2015) investigated the inhibitory effects of camel milk against *S. aureus* and *E. coli* in Wistar rats and concluded camel milk as a beneficial antibacterial food supplement. Camel milk with ciprofloxacin (antibiotic) showed synergistic effect against pathogens and reduced bacterial resistance and antibiotics dose.

Minimum inhibitory concentration (MIC)

This study was planned to determining MIC of CLf against *E. coli* and *S. aureus,* the two most important food borne pathogens. The MIC for CLf came out to be 0.5 mg/mL against *E. coli,* and 0.2 mg/mL in case of *S. aureus.* These findings could be helpful in further studies regarding the utilization of Lf in bio-preservation. Conesa and his group (Conesa *et al*., 2008) determined the MIC of CLf for *E. coli* at 24 and 48 h. According to Conesa *et al*. (2008), the MIC value was 0.5 mg/mL at 24 h and 1 mg/mL at 48 h. These results reflected that the Lf was well existing in mammalian species with subtle differences in their structure leading to variation in their antibacterial activity. In order to study the trend of microbes versus Lf, the workers also examined Lf from different mammalian species. Tomita and his colleagues (Tomita *et al*., 1991) reported the MICs for undigested bovine Lf against *E. coli, Listeria monocytogenes* and *Salmonella* as 2.1, 1.6 and 8.0 mg/ml, respectively which showed that mostly *E. coli* was susceptible to be inhibited at lower dose (2.1 mg/mL) as compared to *Salmonella* (8 mg/mL) for its inhibition. Their findings supported our results illustrating the trend of inhibiting *E. coli* at lower doses. Some other studies done later (Kutila *et al*., 2003) with the same objective were also of the same view.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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