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Development of New Strategy for Non-Antibiotic Therapy: Dromedary Camel Lactoferrin Has a Potent Antimicrobial and Immunomodulator Effects*

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ABSTRACT

The human and bovine lactoferrin have been studied extensively, but very few reports have been published concerning camel lactoferrin (cLf). The present study aimed to isolate cLf and evaluate its efficiency including antimicrobial activity and immunomodulator effects. cLf isolation was attempted from camel milk whey using a cation exchange chromatography by SP-Sepharose. The antimicrobial activity of the isolated cLf was investigated against *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* (*S. agalactiae*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aerogenosa*) strains. The immune effect of cLf was studied by lymphocyte transformation test. It was found that cLf was separated around molecular weight of 80 kDa and showed significant inhibitory effect against *E. coli* followed by *P. aeruginosa*, *S. agalactiae* and *S. aureus*. cLf increased lymphocyte transformations mean values in a dose dependant manner. The highest transformations mean value was determined at 50 µg/mL. In conclusion, these results suggest that cLf is a potent natural antimicrobial and novel immunomodulator agent.

Keywords: Dromedary Camel Lactoferrin; Isolation; Antimicrobial and Immunomodulator Effects

1. Introduction

Few studies have been reported on camels and camel milk [1]. Dromedary camel milk and their products are a good nutritional source for the people living in the arid and urban areas. In addition, fresh and fermented camel milk were reported to provide particular health benefits to the consumer depending on the bioactive substances in milk [2]. Antibiotics are commonly used for both prophylaxis and treatment of various bacterial infections in human and farm animals. In recent years, antibiotics resistance in bacteria of animal origin and its impact on human health have drawn much attention worldwide [3]. Bovine mastitis is the most common cause for the use of antibiotics agents in lactating dairy cattle [4] and the detection of antibiotics residues in milk poses health hazards to consumers, and the cause of high economic importance because such milk is unfit for processing and subsequent consumption [5]. Moreover, the antibiotic therapy has many complications as hypersensitivity, direct toxicity, antibiotic-induced immunosuppresion and super-infections. This is highlighting the need for a new strategy for non-antibiotic therapy using novel immunomodulators as naturally released immunomodulators (Lactoferrin (Lf), cathelicidins and defenses) or bacterial products (Periplasmic proteins and lipopolysaccharides).

Lactoferrin (Lf), is an iron-binding glycoprotein found in a variety of body secretions including tears, bronchial mucus, and saliva and it is found in high concentrations in the mammary secretions of nonlactating dairy animals. It is important in regulation of iron metabolism [6]. This natural antimicrobial agent is a multifunctional bioactive molecule with a critical role in many important physio-

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logical pathways. Lf could elicit a variety of inhibitory effects against microorganisms, comprising stasis, cidal, adhesion-blockade, cationic, synergistic, and opsonic mechanisms. Broad-spectrum activities against different bacteria, viruses, fungi, and parasites, in combination with anti-inflammatory and immunomodulatory properties, make Lf a potent innate host defense mechanism [7]. The large potential applications of Lf have led scientists to develop this nutraceutical protein for use in feed, food and pharmaceutical applications.

Camel lactoferrin (cLf) purification, biochemical, and immunological characterization have shown its similarity to human and bovine Lf, as well as the cross-react with the anti-human Lf antibodies [8-10]. The amounts of lactoferrin and immunoglobulins were found to be greater in dromedary camel milk than bovine or buffalo milk [8,10,11]. Incubation of human leukocytes with cLf leads to a complete virus entry inhibition after seven days' incubation. Thus, cLf markedly inhibits hepatitis C virus genotype 4 infection of human peripheral blood leukocytes [12]. The miR-214 is directly involved in Lf expression and Lf mediated cancer susceptibility (proapoptotic activities) in mammary epithelial cells [13].

Many processing technologies have been developed to isolate the high purity fraction of Lf. And most of the technologies use a cation exchange chromatography on SP-Sepha-rose [14,15].

The aim of this investigation was mainly to isolate cLf from camel milk whey and evaluate its efficacy *in vitro* including antimicrobial and immunomodulator effects. We use cLf but not bovine Lf because cLf is more bioactive [16].

2. Materials and Methods

2.1. Isolation of Lactoferrin from Camel Milk Whey

Lactoferrin (Lf) isolation was attempted from camel milk whey. It was purified using a cation exchange chromatography on SP-Sepharose following the procedure that previously described [14]. Briefly, milk whey was obtained from camel milk using ultra speed centrifuge, 15000 × g at 4˚C for 30 min. Skimmed milk was then diluted 1:1 with the dilution buffer (0.04 M NaH_2PO_4, 0.8 M NaCl, 0.04% (v/v) Tween 20, pH 7.4) and it was incubated with SP-Sepharose at 4˚C overnight. Afterwards, the SP-Sepharose was washed with the washing buffer (0.02 M NaH_2PO_4, 0.4 M NaCl, 0.02% (v/v) Tween 20, pH 7.4) to elude the unbound proteins. The gel then packed into a column (5 × 30 cm or 3 × 30 cm, depending on the milk volume) and lactoferrin was eluted with the elution buffer (0.02 M NaH_2PO_4, 1 M NaCl, pH 7.4). The column was run at a flow rate of 3 mL/min.

2.2. Electrophoresis of Milk and Fractions Containing Lactoferrin

Purity control and characterization of camel Lf (cLf) was done using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Collected fractions of camel milk whey and broad range protein ladder (Fermentra SM1841) were resolved in 12% polyacrylamide minigel-protein II electrophoresis cell (Bio-Rad). Samples were diluted in sample buffer 2-mercaptoethanol (Sigma Chemical Co.), boiled for 5 minutes before being loaded in the gels and run at 70 volts for 3 hours. Gels were stained with 1% Coomassie blue R-250 (Sigma Chemical Co.), then distained at room temperature in 5% methanol and 7.5% acetic acid with shaking for 30 minutes. The different fractions were quantified using Bio-Rad GS 700 imaging densitometer molecular analysis software against broad range marker [17].

2.3. Antimicrobial Activity Assays

Escherichia coli (*E. coli*), *Pseudomonas aeruginosa* (*P. aerogenosa*), *Staphylococcus aureus* (*S. aureus*) and *Streptococcus agalactiae* (*S. agalactiae*) isolates were used to study the antimicrobial activity of cLf. The tested microorganisms were kept in their specific soft agar. Working cultures were obtained by growing the tested isolates on their specific media. After an overnight incubation, an isolated colony was transferred to 10 mL of Mueller-Hinton broth (MHB, Difco Laboratories, Detroit, MI) and incubated at 37˚C for 16 - 20 h. Final concentration of 1×10^6 CFU/mL was used. A volume of 1 mL of cLf solution in different concentrations (1 and 3 mg/mL) was added to 4 wells of tissue culture plates (NUNC. A/S, Roskilde, Denmark) for each of tested microorganisms as previously described [18]. The tested microorganisms in phosphate buffer saline (PBS, 10 mM, pH 7.4) was used a control. Plates were incubated at 37˚C. Aliquots were removed after 1, 3, 6, 12, 24 hours and ten serially diluted, then plated at 37˚C on Mueller Hinton agar (MHA, Difco Laboratories, Detroit, MI) to be counted after 48 h incubation. Total aerobic bacterial count (TBC) of tested microorganisms was done in which viable aerobic mesophlic bacteria were determined as previously described [19]. All equipments used were either sterile new glass or plastic to avoid iron contamination. All experiments were repeated at least two times.

2.4. *In Vitro* Lymphocyte Proliferation Studies

Lymphocyte proliferation test using MTT (3-(4, 5-dimethyl thiazol-2-yl) 2, 5-diphenyl tetrazolium bromide) was performed [20] with modification. Briefly, heaprinized calf blood samples were aseptically collected

in sterile tubes. The separation of lymphocytes was done by layering of blood in Ficol (2:1) and centrifuged at 400 × g at 4˚C for 30 minutes to give packed blood cells with granulocyte, interface layer (which contain lymphocytes) and upper plasma layer. The interface layer was carefully aspirated using sterile glass Pasteur pipette, then placed in sterile tubes containing 2 mL RPMI 1640 medium. Cells were washed 3 times with RPMI 1640 medium by centrifugation at 400 × g for 10 min at 4˚C. After the last wash, the sediment lymphocytes were resuspended in 1 mL of RPMI 1640 medium containing 10% fetal calf serum (FCS). RBCs contamination, if any, was removed by the distilled water lysis method. Lymphocytes were seeded in triplicate in flat-bottom 96-well micro titer plates (Costar) at 1×10^6 cells per well in 150 µL of culture medium either alone or with various concentrations of cLf (10 µg/mL, 20 µg/mL and 50 µg/mL) or 15 µg of Phytohemagglutinin (PHA) control per mL. Another 100 µL of cell suspension was added to three sets of triplicate wells of a RPMI-1640 containing different concentration of cLf (10 µg/mL, 20 µg/mL and 50 µg/mL) plus 50 µL PHA in conc. of 15 µg/mL. The plates were incubated for 3 days under 5% CO_2 at 37˚C. Then 100 µL of supernatant was removed from the wells and 10 µL of MTT solution was added to all the wells. The plate was incubated further for 4 h at 37˚C. The MTT formazon was extracted from the cells using dimethyl-sulphoxide (100 µL/well). Then the OD was taken using an ELISA reader at a test wavelength of 570 nm. All experiments were repeated at least two times.

2.5. Statistical Analysis

The Statistical Products and Service Solutions (SPSS) program was used for all analysis [21]. Data were expressed as mean ± standard error (SE). Comparisons were tested using an analysis of variance (ANOVA) test. A difference was considered to be significant at $P < 0.05$.

3. Results

3.1. Isolation and Characterization of Camel Lactoferrin

The results revealed that the cLf was separated around molecular weight of 80 kDa (**Figure 1**).

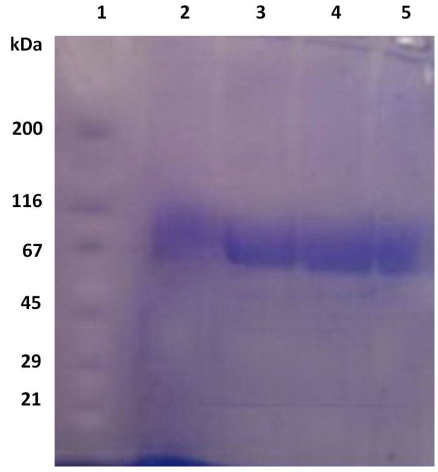

Figure 1. SDS-PAGE of various fractions of Lf purification from camel milk whey. Lane 1, Molecular weight marker; lane 2, Lf standard; lane 3-5, fractions eluted from SP-Sepharose.

3.2. Antimicrobial Effect of Camel Lactoferrin

The antimicrobial activity of the isolated cLf was investigated against *Streptococcus agalactiae* (*S. agalactiae*), *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aerogenosa*) strains. The cLf showed significant inhibitory effect against *E. coli* followed by *P. aeruginosa*, *S. agalactiae* and *S. aureus* (**Table 1**). The inhibition of growth by cLf was concentration-dependent in which a significant inhibitory effect of *E-coli* was observed in a conc. of 1 mg/mL of cLf after 3 h and at conc. of 3 mg/mL after 1 h of incubation. Severe inhibition of growth was observed against *P. aerogenosa* and *S. agalactiae* at conc. of 3 mg/mL after 6 h and 12 h of incubation respectively. *S. aureus* showed slight inhibition of growth at conc. of 3 mg/mL in compared to control.

3.3. Immunomodulator Effect of Camel Lactoferrin

The immune effect of cLf was studied by lymphocyte transformation test (LTT). Phytohemagglutinin (PHA) was used as a control. The obtained results showed that the lymphocyte transformation mean value of PHA was 2.37 ± 0.06 (**Table 2**). While the lymphocyte transformations mean values of cLf alone at concentrations of 10 µg/mL, 20 µg/mL and 50 µg/mL were 1.805 ± 0.040, 1.955 ± 0.045 and 2.39 ± 0.053 respectively (**Table 2**). The cLf increased lymphocyte transformations mean values in a dose dependant manner. The highest transformations mean value was at concentration of 50 µg/mL. On the other side, the lymphocyte transformation mean values of cLf with PHA, at concentrations of 10 µg/mL, 20 µg/mL and 50 µg/mL were 2.12 ± 0.03, 1.941 ± 0.024 and 1.861 ± 0.1 respectively (**Table 2**). This means cLf decreased lymphocyte transformations mean values in a dose dependant manner.

4. Discussion

Lactoferrin (Lf), in this work, was isolated and purified from camel milk whey using a cation exchange chroma-

Table 1. Antimicrobial effect of camel lactoferrin (cLf) on *E-coli, P. aeruginosa, S. aureus* and *S. agalactiae* counts after 1, 3, 6, 12, 24 hours of incubation.

Items		Microbial count (CFU/mL) after				
		1 hour	3 hours	6 hours	12 hours	24 hours
E-coli count	Control	49.000	370.000	2.9×10^6	3.1×10^7	2.7×10^7
	cLf (1 mg/mL)	35.000	15.000	8000	500	CIG
	cLf (3 mg/mL)	CIG	CIG	CIG	CIG	CIG
P. aeruginosa count	Control	1.8×10^4	2.3×10^4	1.7×10^5	2.4×10^6	2.9×10^7
	cLf (1 mg/mL)	142.000	111.000	43.000	21.000	17.000
	cLf (3 mg/mL)	107.000	93.000	17.000	950	950
S. aureus count	Control	87.000	2.3×10^6	2.7×10^7	2.9×10^8	2.2×10^8
	cLf (1 mg/mL)	73.000	2.1×10^6	2.6×10^7	2.7×10^8	2.1×10^8
	cLf (3 mg/mL)	56.000	1.7×10^6	2.0×10^7	2.1×10^8	1.9×10^8
S. agalactiae count	Control	0.7×10^6	2.6×10^6	3.4×10^7	2.9×10^8	3.6×10^8
	cLf (1 mg/mL)	0.4×10^6	1.8×10^6	2.3×10^5	1.8×10^4	2.1×10^5
	cLf (3 mg/mL)	2.2×10^5	1.9×10^5	1.0×10^4	1000	3300

cLf: Camel lactoferrin; **CIG**: Complete inhibition of growth; **N.B.**: *S. agalactiae* was more diluted to be easily counted.

Table 2. Immunomodulator effect of camel lactoferrin (cLf) using lymphocyte transformation test (LTT).

Items	PHA alone	Camel lactoferrin alone			Camel lactoferrin with PHA		
		10 µg/mL	20 µg/mL	50 µg/mL	10 µg/mL	20 µg/mL	50 µg/mL
LTT means ± SE	2.37 ± 0.06	1.805 ± 0.040 ($P < 0.05$)	1.955 ± 0.045 ($P < 0.05$)	$2.39 \pm 0.053^{n.s}$	2.12± 0.03 ($P < 0.01$)	1.941 ± 0.024 ($P < 0.001$)	1.861 ± 0.1 ($P < 0.001$)

PHA: Phytohemagglutinin; **n.s**: non-significant.

tography on SP-Sepharose. Compared to the bovine species, camel whey contains a higher content of antimicrobial factors such as lysozyme, lactoferrin and immunoglobulins [8-10]. Variation in the composition of whey proteins from camel (*Camelus dromedarius*) colostrum and milk was recorded [22] and shown to be rich in protective proteins, especially lactoferrin, peptidoglycan recognition protein and immunoglobulins IgG_2 and IgG_3. Due to Lf large potential applications, many processing technologies have been developed to isolate high purity fractions. Cation-exchange chromatography is already used for the production of Lf at industrial scale [14,16]. This technology has the advantage of producing Lf with a high degree of purity (>90% dry basis). The limitation of this technology for large-scale applications lies with its high cost and its relatively low yield [23]. Characterization of camel Lf (cLf) was done using reduced polyacrylamide gel electrophoresis (SDS-PAGE). cLf was separated around molecular weight of 80 kDa. However, affinity membranes with immobilized triazinic dyes have not achieved yet good acceptance in the biotechnological industry, mainly because of their low capacity for proteins in comparison with the same legends immobilized on soft gels [24] and the dye leaching in the elution and regeneration steps [25]. Although, under equilibrium conditions, membranes show an acceptable chromatographic performance for Lf purification from bovine colostrums, better than the obtained with d-Sepharose, as a model of soft gels [26], the main problems affecting industrial utilization of adsorptive dye membranes, such as low capacity, dye leaching and pressure drop along the fiber axis need to be overcome. On the other side, the recovery of Lf from whey is a relatively difficult task, because not only the huge volume of whey needs to be dealt with, but also the major proteins complicate the separation process [27].

The cLf showed significant inhibitory effect against *E. coli* followed by *P. aeruginosa*, *S. agalactiae* and *S. aureus*. One of the first antimicrobial properties discovered for Lf was its role in sequestering iron from bacterial pathogens as in case of *S. aureus* [28] which is known to be resistant to antimicrobials. It was later demonstrated that Lf's bactericidal function has been attributed to its direct interaction with bacterial surfaces [29] and through an iron-independent mechanism [30] as in case of *E. coli* [31]. Biofilm formation, which was pro-

posed as a colonial organization adhesion method for *P. aeruginosa*, is a well-studied phenomenon. Through biofilm formation, bacteria become highly resistant to host cell defense mechanisms and antibiotic treatment [32]. It is well known that some bacterial strains require high levels of iron to form biofilms. Thus, Lf's function as an iron chelator has been hypothesized to effectively inhibit biofilm formation through iron sequestration [33]. Occurrence in various milieus strongly emphasizes the significance of the structure-function relationship in the multifunctionality of the Lf [7].

Regarding the immune effect of cLf, the cLf increased lymphocyte transformations mean values in a dose dependant manner. The highest transformations mean value was of lactoferrin in conc. of 50 µg/mL. This finding was agreed with [34] who reported that the addition of recombinant human lactoferrin (Talactoferrin Alfa (TLf)) to human peripheral blood or monocyte-derived dendritic cell cultures resulted in cell maturation, as evidenced by up-regulated expression of CD80, CD83, and CD86, production of proinflammatory cytokines, and increased capacity to stimulate the proliferation of allogeneic lymphocytes. In addition, this finding was agreed to some extend with [35] who found that the effects of Lf in experimental models were differential and dependent on an individual PBMC reactivity, mitogen or alloantigen and Lf concentration. Generally, lymphocytes from donors responsive to Lf exhibited higher proliferation indices to PHA when compared with non-responsive individuals, suggest that the differential action of Lf might be due to its ability to sense the activation status of lymphocytes, although he mentioned that data on Lf effects on mitogen-induced proliferation are scarce, though fairly consistent both in the mouse and human systems. It has been demonstrated that human and bovine lactoferrin inhibit proliferative responses *in vitro*.

In addition, the cLf decreased lymphocyte transformations mean values in a dose dependant manner when combined with PHA. This opinion goes hand in hand with [36] who reported that purified lactoferrin, isolated from human milk, was tested for its effect on human T-lymphocyte proliferative responses to Phytohemagglutinin (PHA) and to alloantigen in mixed lymphocyte culture. Lf inhibited proliferation in both assays in a dose-dependent manner. The suppressive effect was not due to Lf mediated cytotoxicity since washing cells that had been pre-incubated with Lf restored their proliferative activity. Lf was most effective in suppressing the PHA response when added within 24 h of culture initiation. Iron saturated Lf failed to inhibit PHA-induced proliferation, suggesting that the mechanisms of suppression involve the chelating property of Lf. The suppressive effect of Lf on T-lymphocyte proliferative response *in vitro* supports the notion that Lf has significant immunoregulatory potential *in vivo*. The same agreement was concluded by [35] that the effects of Lf on the proliferative response of lymphocytes to PHA were generally stimulatory at lower and inhibitory at higher concentrations of Lf. The increased production of cytokines may play a significant role in the down-regulation of mitogen-induced lymphocyte proliferation in the presence of Lf.

5. Conclusion and Recommendation

In conclusion, these results suggest that cLf is a potent natural antimicrobial and novel immunomodulator agent. The extensive uses of Lf in the treatment of various infectious diseases in animals and humans have been the driving force in Lf research, however, a lot of work is required to obtain a better understanding of its activity. Further studies will be needed for molecular cloning, promoter analysis and identification of camel lactoferrin gene.

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