Lactoferrin, Isolation,Purification and Antimicrobial Effects

Fatemeh Moradian
Basic sciences group, Sari Agricultural Sciences and Natural Resources University,
P.O.B. 578, Sari, Mazandaran, Iran
E-mail: moradi_f@yahoo.com

Ramisa Sharbafi and Alireza Rafiei
Molecular and Cell Biology Research Center, Mazandaran University of Medical Sciences

***Abstract*—Lactoferrin (Lf) is an 80 kDa iron-binding glycoprotein with multifunctional properties and useful for clinical and commercial applications. Currently, Lf has some advantages including, immune system modulation, antibacterial activity and as antioxidant in infant and adult of human as well as animal health. In the present study, antibacterial activity of Lf has been scrutinized after isolation and purification from cow's milk colostrums against *Pseudomonas aeroginosa.* Lf purified using CM-sephadex C50, a cation exchange chromatography. Bacterial samples were isolated from scald patients and microbial activity was confirmed by biochemical tests. Different concentration of Lf (0, 200, 400, 500, 600 and 700 µg/ml) treated on *Pseudomonas* colonies as well as *E.coli*(DH5α) as positive control for two days. Our result indicated LF was effective on *Pseudomonas* growth and the least and the inset inhibitory concentration were 400,and 700 µg/ml, respectively. The result showed that The Lf has more strong effect than other previous studies.**

Index Terms*— antimicrobial, cation exchange, lactoferrin, *pseudomonas.

I. Introduction

Lactoferrin is an 80 kDa iron binding glycoprotein of the transferring family. Lf is a major component of milk and presents in neutrophil granules or other exocrine secretions such as tears, saliva and the servical mucus. Lactoferrin is considered to be an important host defence molecule and has a diverse range of physiological functions such as antimicrobial, antiviral and anticancer and so on, activities. During the past decade, it has become evident that oral administration of Lf exert several beneficial effects on the health of human and animals, including: anti-infective, anticancer and anti-inflammatory effects. Recently it has been recognized that oral administration of Lf exerts various health beneficial effects such as anti-infective activities not only in infants but also in adult animals and human [1]. Lf is capable of retarding the growth of certain microorganism [2]. The antibacterial activity of Lf has been documented in the past, both in vitro and in vivo for Gram positive and Gram negative bacteria [3]. Since this inhibition is readily reversed by the addition of iron in excess of the binding capacity of the lactoferrin, it has been suggested that Lf stasis may be due to its ability to withhold iron that is essential for bacterial growth [4]. The sequestration of iron away from bacterial pathogens inhibits bacterial growth, limits the use of tis nutrient by bacteria at the infection site [5] In addition to this bacteriostatic effect, it has been shown that Lf is capable of a direct bactericidal effect on *Streptococcus mutans* and *Vibrio cholerae* [6]. Lf's bactericidal function has been attributed to its direct interaction with bacterial surfaces. In 1988 it has shown that Lf damages the external membrane of Gram negative bacteria through an interaction with lipopolysaccharide (LPS) [7] *Pseudomonas aeroginosa* is a common bacterium which can cause disease in animals and human. It is found in soil, water, skin flora and most environments throughout the world. It is an opportunistic pathogen that infects burned patients with immunological system defect. Burn injury is one of significant public health problems in many area of the world. Since *P. aeroginosa* in naturally resistant to many drugs and is able to gain resistance to all effective antibiotics, the infection with this organism is a particularly problem for patients [8]. In this study, antibacterial activity of Lf examined on *Pseudomonas aeroginosa* as well as *Ecoli* and the study of mode of Lf action on these bacteria.

II. Materials and Methods

A. Isolation and Purification of Lactoferrin

Colostrum of cows purchased from faculty dairy farm of behshahr.At first the cream was separated by centrifugation (10000 ×g, 20 min at 4 °C). Then casein removed from skim milk in acidic condition using 2N HCl incubated at 40 °C for 30 min.The acid whey was neutralized to pH 6.8 with 2N NaOH. Some extra proteins precipated using ammonium sulfate in two steps and after centrifugation in 10000×g ,30 min at 4℃ , the precipitate was dissolved in 20 mM phosphate buffer then diafilteration finally, the protein powder obtained by freeze drying. Lf has a cationic nature according to its amino acids composition thus it can be purified by cation

Manuscript received June 16, 2013; revised August 25, 2013.

doi: 10.12720/jomb.3.3.203-206

exchange chromatography. Lactoferrin was purified by carboxymethyl Sephadex-C50 chromatography(FPLC, Bio-RAD, USA) using 0.2 M phosphate buffer (pH7.7) and linear gradient NaCl from 0.0 to 0.5 M. During chromatography, protein in the eluents was monitored by ultraviolet absorption at 280 nm with the instrument[9].

B. Cell Growth and Antimicrobial Assay

Pseudomonas samples were isolated from burnt patients (Shahid Zareh Hospital,Sari) and confirmed by biochemical and microbiology tests such as, oxidase, catalase as well as culture in specific media such as TSI and mueller Hinton agar. In order to determination of CFU, serial dilution of microbial culture (10^1 to 10^{12}) prepared then 100 µl of each dilution was plated onto EMB plate and incubated at 37℃ for 18 h after that colonies were counted on the plate. Bacterial suspention were selected to the final concentration of 1×10^3 CFU/ml for experiments. Different concentrations of Lf treated on bacteria growth in EMB media and incubated at 37 ° C for 18 h. *Ecoli* strain was used as standard positive control as well as bacteria in the absence of Lf used for negative control. The colonies were counted and colony forming unit (CFU) determined. All experiments replicated 4 times.

III. Result and Discussion

A. Purification of Lactoferrin

Lactoferrin was released from 0.4 to 0.5 M of NaCl linear gradient. A strong peak was observed between 0.4 to 0.5 M (Fig. 1).The single band of purified Lf has been observed in SDS-PAGE electerophoresis(data was not shown). The concentration of Lf determined by Brad ford assay and was about 2.4 mg/ml.

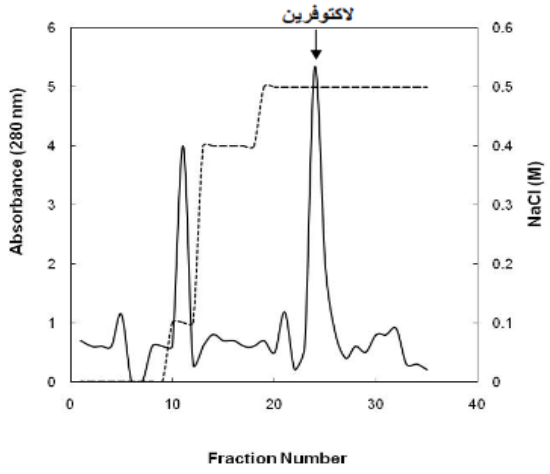

Figure 1. Purification graph of Lf, first peak is lactoperoxidase that eluted in 5-10% of 1M NaCl concentration. The strong peak is Lf that eluted in 40-50% of 1 M NaCl.

Purified Lf in the present study had very good concentration than volume of milk and also purified in one step with biological activity and purification efficiency was about 90%. The mentioned method, apart from simplicity and speed, can result in isolation of highly pure lactoferrin. However in previous study showed that single step purification by affinity chromatography but Lf obtained without activity and needed reactivity by some procedure[10].

B. Antibacterial Activity

The effect of Lf on bacterial growth were examined with different concentration of Lf (0,400,500,600700 µg/ml) in 18h of incubation. The result indicated that 400 µg/ml had the least inhibitory effect with 35% growth inhibitory where as maximum inhibitory concentration was 700 µg/ml with 86% inhibitory effect on *Pseudomonas*. Also the same result was shown in *Ecoli* but the inhibitory effect of Lf on *Pseudomonas* was more than *Ecoli*. In *Ecoli* DH5α with 29% and 66% growth inhibitory for minimum and maximum inhibition, respectively (Fig. 2)

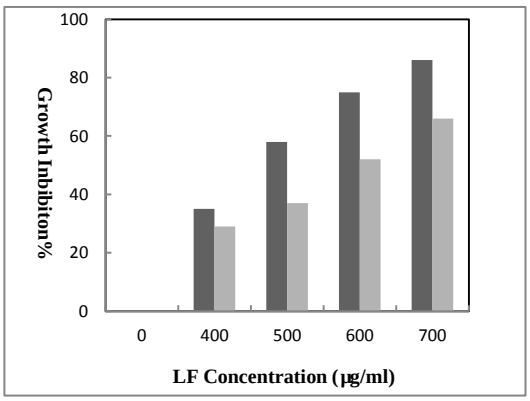

Figure 2. Graph of different Lf concentrations on growth of bacteria.The blak column is *Pseudomonas aeroginosa* with minimum and maximum inhibitory effect about 35 and 86%, respectively and the grey column is *Ecoli DH5α* with minimum and maxmimum inhibitory effect about 29 and 66%, respectively.

Antimicrobial activity of Lf against *Pseudomonas aeroginosa* may be explain by several mechanism. Th first mechanism is that Lf is an iron-binding protein which scavenger free iron and reduce in the environment of microorganism.Thus deficiency of iron prevents biofilm formation by *Pseudomonas*. Biofilm formation which was proposed as a colonial organization adhesion method for *Pseudomonas aeroginosa* is a well-studied phenomenon in patients suffering from cystic fibrosis. Through biofilm formation bacteria become highly resistant to host cell defense mechanism and antibiotic treatment [11].It is well known that some bacteria stains require high level of iron to form biofilms. Thus Lf's function as an iron chelator has been hypothesized to effectively inhibit biofilm formation through iron sequestration [12].

The second mechanism is suggested that lactoferrin with binding to the lipid A causes discontinuity membrane of gram-negative bacteria resulting destabilizing the outer membrane of the bacteria and release of lipopolysaccharide (LPS), and ultimately can be lead to changes permeability in the membrane [13]. Rceptors for the N-terminal region of Lf have been discovered on the surface of some microorganisms. The binding of Lf to these receptors induces cell-death in Gram negative bacteria due to disruption in the cell wall

[14]. The positively charged N-terminus of Lf prevents the interaction between LPS and bacterial cations (Ca^{2+} and Mg^{2+}) and interferes with aggregative proliferation in *E.coli*. The interaction between Lf and LPS or other surface proteins also potentiates the action of natural antibacterial such as lysozyme which is secreted from the mucosa at elevated concentrations along with Lf [15]-[17]. Both of two mechanisms have been seen in different starins(Table I) [18]-[21].

TABLE I. BACTERIA AGAINST WHICH LF HAD A REPORTED EFFECT

Gram negative starins	Mechanism of action
Ecoli.(DAEC)	Inhibit aggregative proliferation
E.coli entheropathogenic	Inhibits adherence of diffuse adherent
Pseudomonas aeroginosa	Prevents biofilm formation
Samonella enteritidis	Interferes with polysaccharide cell content
Mycoplasma bovis	Prevents biofilm formation

IV. CONCLUSION

The results of this study demonstrate that all concentration of bovine lactoferrin significantly inhibits the growth of *P. aeroginosa* and *Ecoli*. The effect of lactoferrin was more effective than previous study since minimum and maximum inhibitory effect of LF was less [8], [22]. The result suggested that Lf had bacteriostatic effects because bacteriocidal activity is not iron dependent. The results indicate that the incorporation of bovine lactoferrin is expected to protect dairy products, food and fruits from pathogenic bacteria.

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F. Moradian was born in Ghaemshahr, Mazandaran, Iran, 2/8/ 1973.

She received B.Sc., Biology, University of shiraz, Shiraz, Iran, 1996, and Ms, Biochemistry, University of Tarbiat Modares, Tehran, Iran, 2000; PhD, Biochemistry, University of Tarbiat Modares, Tehran, Iran, 2006. She has teaching experience in Biochemistry, Enzymology, Biochemistry mechanism of Cancer, cell culture and Bioinformatic at Sari Agricultural Sciences and Natural Resources University, ,Sari, Iran, 2006- 2013.

The major field of study is enzymology, protein purification, molecular cloning, cell biology and cancer research.

Jobe title: ASSISTANT PROFESSOR in Basic Sciences Group, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.

Three publication:

1) F. Moradian, C. Garen, L. Cherney, M. Cherney, and M. N. G. James, " Expression, purification, crystallization and preliminary x-ray analysis of two arginine-biosynthetic enzymes from Mycobacterium tuberculosis," *Acta Crystallographica*, F62, pp. 986-988, 2006.
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