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REVIEW ARTICLE

Elucidation of Lactoferrin and Lactoperoxidase in Separation Technique

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ABSTRACT

This review confers the biological properties of the glycoproteins lactoferrin (Lf) and lactoperoxidase (Lp). Lf is an iron-binding protein present in huge amounts in colostrum and breast milk, in external secretions, and polymorphonuclear leukocytes. Lp is a member of a large group of mammalian heme peroxidases that originates in exocrine secretions including milk. Lf holds numerous biological functions that contain roles in iron metabolism, cell proliferation, and differentiation, antibacterial, antiviral, and antiparasitic activity. Lp is accountable for the inactivation of an extensive range of microorganisms and hence significant in the defense mechanism in human secretions such as saliva, tear fluid, and milk. Lately discovered is the anticancer activity. Extraction of Lf and Lp can be done from raw milk by cation exchange chromatography. Separation and purification technologies can be achieved from whey and bovine milk by batch extraction, chromatographic techniques, and with hydrophobic ionic liquids. Polyclonal antibodies were made to purified breast milk Lf and used in an ELISA to estimate plasma concentrations in investigations of innumerable aspects of the inflammatory reaction. The usage of Lp system in the dairy industry and the possible applications of the Lp system in further food systems and commercial products are emphasized here. Milk Lf is used as a dietary constituent that endorses growth of gastrointestinal tract of human infants and newborn nonhuman animals instantaneously on birth. The paper as well highlights the investigation breach and promising forthcoming research directions that require consideration.

Keywords: Anticancer, chromatography, dietary, ELISA, glycoprotein.

INTRODUCTION

Lf

Lactoferrin (Lf) is a globular glycoprotein with a molecular mass of about 80 kDa that is extensively represented in numerous mucosal secretions; such as milk, saliva, tears, nasal and bronchial secretions, vaginal fluids, semen,[1] bile, gastrointestinal fluids, and urine.[2] Lf was first isolated by Sorensen and Sorensen from bovine milk in 1939, and after two decades it was determined to be the main iron-binding protein in human milk.[3,4] Lf is formed and released by mucosal epithelial cells and neutrophils in several mammalian species, including humans, bovines, cows, goats, horses, dogs, and several rodents. Neutrophils after degranulation were observed to be the main source of Lf in blood plasma. Lf can also be purified from milk or produced recombinantly. Human colostrum ("first milk") has the peak concentration, which is followed by human milk, then cow milk (150 mg/l). Lf is an iron-binding glycoprotein which belongs to the transferrin protein family. Lf is found to be involved in several physiological and protective purposes, including regulation of iron absorption in the bowel, antioxidant, anticancer, anti-inflammatory, and antimicrobial activities.

Lp

Lp (E.C. 1.11.1.7) is a heme-containing chain glycoprotein which is found in milk and further exocrine secretions such as saliva, tears, and airways. It belongs to the family of mammalian

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heme-containing peroxidase (XPO) enzymes. Lp was named so because it was isolated from milk in crystalline form for the 1st time as per the journal by Morrison *et al*.[5] The protein was also found to be secreted from other glands, such lacrimal glands, hardenian glands,[6] and salivary glands.[7] Their Prime Task is to catalyze the oxidation of certain molecules, at the expense of hydrogen peroxide, to produce reactive products with widespread antimicrobial activity. In bovine milk, it is one of the unique indigenous antimicrobial agents. Not only that, antiviral action, degradation of numerous carcinogens, and fortification of animal cells against peroxidative properties have also been stated.

STRUCTURE

Lf

The molecular structure and amino acid sequence of human Lf were discovered in 1984. The Lf gene (chromosome 3) codes for the manufacture of Lf or delta-Lf (nucleocytoplasmic form) dependent on promoter usage. Lf is comprised a single polypeptide chain which contains 703 amino acids fold into two globular lobes. One polypeptide chain is folded to yield a similar structure to transferrin where two lobes connected by a small peptide chain. The lobes (N and C) each carry a glycosylation site and an iron-binding site and configure differently when carrying iron or glycosylated. These structural modifications elucidate changes in function between apolactoferrin (iron free) and holo-lactoferrin (iron-rich). Each lobe comprises two domains known as C1, C2, N1, and N2. The domains form one iron-binding site on each lobe. Crucial peptide clusters of Lf are recognized to confer the precise functional properties of iron sequestration, nuclear targeting, lipopolysaccharide (LPS) binding, modulation of inflammatory response, antimicrobial activity, and stimulation of apoptosis, etc. Lf is subjected to proteolysis, and certain essential functions are transported by the cleaved fractions: Lactoferricin which is 25 amino acids long, structurally reformed to configure as a beta-sheet, which has improved interaction with microbes and is accountable for many "direct" antimicrobial properties of Lf.

Lf -α is the iron binding form but has no ribonuclease activity. On the other hand, Lf -β and Lf -γ demonstrate ribonuclease activity, but they are not able to bind iron [Figure 1].

Lp

The chief structure of Lp from all the species is a single chain monomer of 595 residues that contain a heme moiety in the catalytic center. The complete structure of Lp is oval shaped, exposed on one side by a substrate diffusion channel which leads to the central of the heme cavity and entirely closed on the other side by a tight criss-cross of α-helices. The core features of the structure of Lp are the 20 α-helices (H1 to H19) which make the essential central part of the structure; two tiny anti-parallel β-strands (S1 and S2) and the central heme cavity which can be distributed into proximal and distal cavities. The proximal heme cavity is noticeably smaller when paralleled to the equivalent distal cavity. It consists of His351 of helix H8 which interacts with the iron atom of the heme. The heme molecule is bound in a heme cavity which is deeply submerged inside the structure of Lp. Five helices (H2, H5, H6, H8, and H12), also called the "core helices," are organized in an exclusive structure which provides the required stereochemistry and shapes for the heme to bind in the heme cavity. The two β-strands, S1 and S2 form the upper side wall of the opening to the heme cavity. Therefore, the iron position is shifted to some extent toward the proximal site. The distal heme cavity, which is greater than the proximal heme cavity, consists of three aspects: The substrate diffusion channel, the substrate binding site, and a well-preserved water network [Figure 2].

Physicochemical properties

Lf

Lf occurs in 3 forms according to its iron saturation: Apolactoferrin (iron free), monoferric form (one ferric ion), and holo-lactoferrin (binds two Fe^{3+} ions). Four amino acid residues are vital for an iron-binding (histidine, twice tyrosine, and aspartic acid), while an arginine chain is accountable for binding the carbonate ion. Moreover, iron lactoferrin is adept of binding a hefty volume of other compounds and substances, for example, LPS, heparin, glycosaminoglycans, DNA, or other metal ions such as Al^{3+}, Ga^{3+}, Mn^{3+}, CO^{3+}, Cu^{2+}, and Zn^{2+}. The capability to retain iron

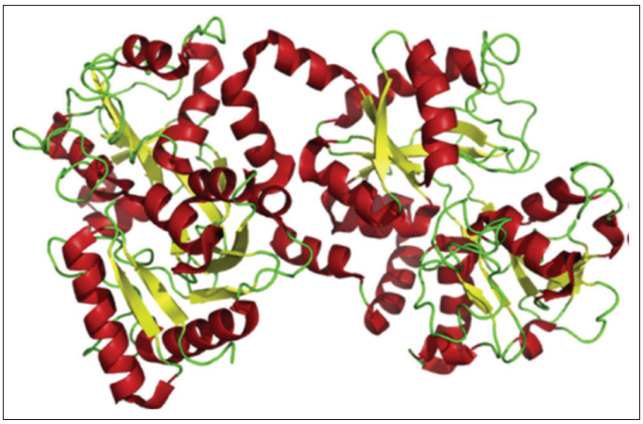

Figure 1: Structure of lactoferrin

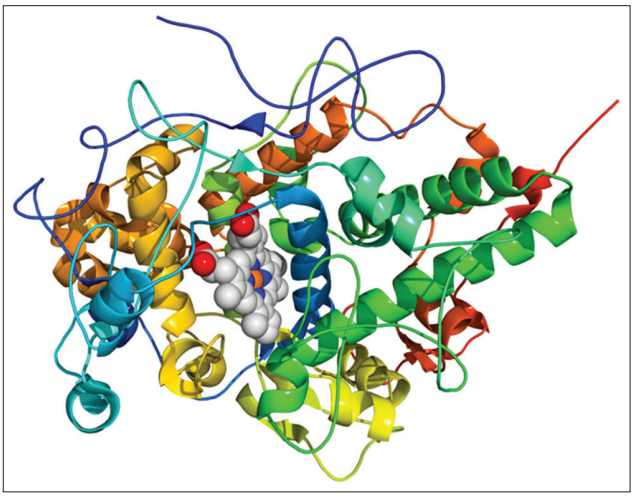

Figure 2: Structure of lactoperoxidase

bound even at low pH is essential, specifically at positions of infection and inflammation where, due to metabolic activity of bacteria, the pH may drop under 4.5. Lactoferrin has established noteworthy resistance to proteolytic degradation by trypsin and trypsin-like enzymes. The level of resistance is proportional to iron saturation. Lf is prone to peptic digestion in the stomach; it cannot easily accessible to the digestive tract. Liposomalization increased the Lf action by the improvement of the stability against gastric degradation and facilitated the interaction with the intestinal membrane and with Lf-specific receptors.[8,9]

Lp

Studies in milk, whey, permeate and buffer exhibited that heat denaturation of Lp starts at temperatures from about 70°C. Lp is less heat stable under acidic conditions (pH 5.3), probably due to the release of calcium from the molecule. Lp is deactivated by storage at pH 3, and some denaturation was observed at pH<4. Lp is somewhat resistant to proteolytic enzymes. In the presence of riboflavin, Lp appears to be very sensitive to light.

EXTRACTION

By chromatographic methods

The method involves the extraction of raw milk with a cation exchange resin using a concentrated sodium chloride solution. A solution of basic milk protein contains Lf, Lp, and other impurities. Purification main milk protein solution onto a cation exchange resin equilibrated with acetate buffer at a concentration of 50 mM at a pH from 4 to 9. Elution was carried out with solutions of acetate buffer concentration of 50 mM at different concentrations of sodium chloride solution - from 0.02 to 1.5 M. A drawback is the fact that the milk used as raw material which is subjected to treatment at a temperature >55°C.

Known purification method of raw milk to obtain Lf consist of adsorption of proteins on ion exchange, chromatography their separation, elution fractions with determining the optical density at a wavelength of 280 nm of more than 0.1 units. Dialysis the protein fraction obtained against saline, phosphate buffer, or distilled water. Microbiological stabilization and purification are carried out by microfiltration through a semipermeable membrane 0.22–0.45 um. The disadvantage of this invention is the relative complexity associated with performing a gradual purification of the protein, as well as the inability to re-use of raw milk for human consumption.

The method includes the purification of raw milk, separation, precipitation of casein, two-stage ultrafiltration, chromatographic separation of proteins, dialysis, microfiltration with a selectivity of pores of 0.2–0.3 μ, the mixing ratio in isolated mono fractions similar svezhevydoennom their content in cow's milk, and liquophilic drying. In the first ultrafiltration step is carried out the allocation of serum immunoglobulin fraction through a 100 kDa membrane. Repeated ultrafiltration through membranes of 30 kDa is subjected to permeate. The obtained protein concentrate is subjected to ion exchange chromatography, NaCl gradient from 0.35 to 1 M to obtain fractions laktoferrin voy and Lp. The disadvantage of this invention is the relative complexity associated with performing a gradual purification of the

Table 1: Physicochemical characteristics of bovine Lp

Characteristic	Data	Reference
Molecular weight	78 431 Da	Paul and Ohlsson, 1985
Amino acid residues	612	Cals *et al*. 1991
Half cystine residues	15	Cals *et al*. 1991
Carbohydrate content	10%	Carlstrom, 1969
Iron content	0.07%	Paul and Ohlsson, 1985
Prosthetic group	haem: protoporphyrin IX Thanabal & La Mar, 1989	
Iso-electric point	9.6	Paul, 1963
Secondary structure	23% α, 65% β, 12% unordered	Sievers, 1980
Absorptivity ε_{412} nm	112.3 mM^{-1} cm^{-1}	Paul and Ohlsson, 1985
Absorptivity 280 nm	14.9 ± 15.0,1%, 1 cm	Paul and Ohlsson, 1985
Redox potential E_m	−191 mV	Paul and Ohlsson, 1985

Lp: Lactoperoxidase

protein, the necessity of separation of ballast proteins, as well as the impossibility of reuse of raw milk for human consumption [Table 1].

A method of obtain Lf from human milk comprising decreasing, removal of casein, ammonium sulfate fractionation, dialysis, and lyophilization the desired product. Fractionation steps are: First, ammonium sulfate was added to a concentration of 35–45% saturation, and the precipitate was removed, then added to the supernatant ammonium sulfate to a concentration of 70–80% saturation, and the precipitate before dialysis by fractionation was dissolved in distilled water and incubated with 1.3–3.7 M sodium chloride at pH 3.6–4.4 and the resulting precipitate was removed by filtration. The closest to the claimed one is a method of obtaining Lf from raw cow's milk, which is passed through the chromatographic column with the sorbent (prototype, patent RU 23902531). The method involves that the use of milk as raw milk Lf chromatography was carried out in a column of carboxymethylcellulose sorbent, equilibrated with 0.05 M sodium phosphate buffer at pH 7,7, eluted Lf carried out with 0.05 M sodium phosphate buffer pH 7,7 in a gradient of sodium chloride concentration of 0.1–1, 2M, eluting fractions Lf solution with an optical density of 0.5 units. At a wavelength of 280 nm, and forward them to a second chromatography with the same parameters, and then fractions isolated are fed to ultrafiltration, desalting, and lyophilization. The method allows to obtain a native Lf purity not <98%. The disadvantage of this invention is the relative complexity associated with performing a gradual purification of the target protein, as well as the inability to re-use of raw milk for human consumption.

By batch extraction methods

The Lf is directly perceived as cellulose phosphate by batch extraction and eluted by a stepped salt and pH gradient. The foremost impurity a low molecular weight fraction is rapidly removed by gel extraction. The recovered Lf has a purity of 96%. The end of Lf averaged 80%. Purification from human milk and the standard sources embraces the preparation of whey and two column chromatographic procedures.[10-13] A main source of Lf impurities seems to be the affinity of Lf to cumulative with other whey proteins such as α-lactalbumin and serum albumin.[14] Whey was prepared from thawed and pooled frozen human milk fractions according to the method of Blackberg and Hernell[10] settled cellulose phosphate in 20 Mm NaH_2PO_4 at pH 7.0 was added at a ratio of 10 ml–100 ml of whey. The mixture was readjusted to pH 7.0 and stirred rapidly for 2 h so that the cellulose phosphate was not able to settle during the extraction period. The cellulose phosphate pad was washed with four successive pad volumes of 0.1M NaCl in 0.1 M NaH_2PO_4 at pH 7.0, again under moderate vacuum. The pink cellulose-phosphate pad was then poured as a slurry into a glass column. Lf was eluted with 0.25 M NaCl in 0.2 M NaH_2PO_4 at pH 7.5. Precaution was taken to sustain a minimal elution volume. The fraction with a UV Absorbant >1.0 was cooled and subjected to gel filtration on Sephadex-100. After gel filtration, Lf bearing fractions with an uv absorbants >1.0 were pooled and dialyzed against 50 Mm NaCl in Mm hepes at Ph 7.4. The Lf was then lyophilized and stored under an anhydrous condition at −20° C. The determination was done in the following ways. A 4.5 ml aliquot of whey was mixed with 0.5 ml of 100 Mm $NaHCO_3$. To a 1.5 ml aliquot

of this, 50 µl of mm Fe^{3+}nitriloacetate was added. After 30 min, in absorbance at 465 nm was read against whey blank. Purified or partially purified Lf samples in 25 mm $NaHCO_3$ were determined by the additional of a two-fold excess of Fe^{3+}nitriloacetate, prepared as termed by Graham and Bates.[15] The absorbance was read after 30 min at 465 mm the molar absorptivity at 465 and 280 nm was determined by titration of Lf in 25 mm nahco3 and 100 mm hepes at pH 7.4 with $Fe(NH_4)_2(SO_4)_2$ in 0.01 m HCl. The absorbance at 280 nm was determined for whey and Lf after dilution in 10 mm hepes, pH 7.4. Approximately 80% of the original Lf was usually recovered. SDS- Gel electrophoresis revealed a low molecular weight fraction to be the major impurity.

By hydrophobic ionic liquids

Lf extraction efficiencies of up to 20% are achieved in this method. The bovine Lf (protein: 98.6%) used in this study. Its iron saturation is equal to 8%, which was measured by means of a 7500ce inductively coupled plasma mass spectrometer. The BSA (protein: 98%≥, code: A7906) and sodium dodecyl sulfate, SDS (05030). The ionic liquid 1-butyl-3methylimdidazolium bis[(trifluoromethyl)sulfonyl]imide (BmimNTF2) and 1-butyl-3methylimdidazolium hexafluorophosphate (BmimPF6) was brought. The reagent required to prepare a buffer solution such as potassium hydroxide, KOH (121515), L(+)- lactic acid, LA(121034) and orthophosphoric acid, H^3PO^4 (131032). Buffer solution at ph 3.2, 6.4, and 8.2 was prepared with H_3PO_4 and KOH whereas LA and KOH were required to prepare a buffer solution at pH 4.6. The 18.2MΩ cm deionized water (Milli-Q, Millipore) was throughout. Method of extraction is to study the protein extraction, and back-extraction has been carried out in a U-shaped tube with an inner diameter of 11.5 mm as shown in Figure 3. Ionic liquid acts as a bulk liquid membrane which separates both sides of the tube. This arrangement makes it likely to assess the achievability of both the protein extraction and back-extraction only considering the influence of ionic liquid and experimental condition on extraction and avoiding the effect of additional variables (e.g., material support when supported liquid membrane is used). One side (A) is filled with the feed solution

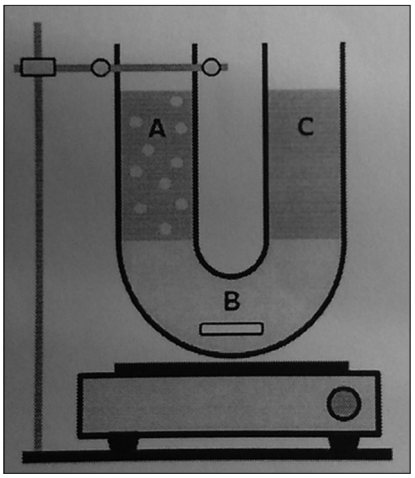

Figure 3: U-shaped tube

prepared with the protein under study whereas the other side (C) contains the stripping solution. The volume of each phase was 7.5 ml the ionic liquid (B) continuously stirred at 300 rpm to assure its homogeneity. Samples of 2.5 Ml were taken at regular intervals to measure the protein concentration of the aqueous solution (A and C); once they were measured, samples were return to the U-tube. For this reason, and due to the hydrophobicity of ionic liquids, it was consider that the volume of phases remained constant, which was confirmed experimentally. All the experiment was carried out at room temperature. The protein content of Lf [59.60] or BSA [5.30] in aqueous phases was determined by measuring the absorbance at 280 nm using a spectrophotometer. In spite of the very low solubility of both ionic liquid in water, for each experiment, another U-shaped tube filled with feed solution without any protein was used as blank to be sure that ionic liquids do not interfere in measurement. Protein concentration in the ionic liquid was calculated by mass balance.

By cation exchange chromatography method

The extraction of high-value dairy proteins such as Lf and Lp generally involves widespread pre-treatments of milk to remove fat and caseins by centrifugation, precipitation, Ca2+ chelation, and/or filtration. Likewise, fat and caseins are normally removed before capture of recombinant proteins from the milk of transgenic animals. Such pre-treatments can result in significant loss of protein yield and/or activity. In this paper, we validate that it is possible to pass major quantities of raw, untreated milk through a 5 cm high XK16

chromatography column (volume 10 mL) packed with SP Sepharose Big Beads TM without beyond the maximum permissible backpressure, provided that the processing temperature is kept nominally around milking temperature (35–37°C). Milk was held after assortment at 37°C under gentle stirring to prevent fat separating under the influence of gravity until it was processed. Holding times were typically <1 h. Affinity purified goat polyclonal anti-bovine Lf antibody (1 mg/mL). Lp standard was achieved from Sigma-Aldrich. SP Sepharose Big Beads TM was used to adsorb Lf and Lp proteins from raw whole milk. Resin was equilibrated before use in 10 mM phosphate buffer (10 mM mono and dibasic sodium phosphate) at pH 6.7. Protein elution was achieved in the same buffer using either gradient (0–1.0M NaCl) or step elutions. Step elutions were carried out in two steps: 0.4M NaCl to elute Lp and 1.0M NaCl to elute Lf. An XK16 water-jacketed chromatography column, connected to an AKTAfplc fast protein liquid chromatography system, was used for all column-based chromatographic milk processing. The column was packed to a height of 5 cm, following the manufacturer's instructions, giving a bed volume of 10 mL. To determine equilibrium isotherms, 0.2 g of equilibrated, swelled, and drained resin was quantitatively weighed into 10mL centrifuge tubes. Lf and Lp standards from samples of known purity were constituted to concentrations ranging from 0.05 to 20.0 mg/mL. 5 mL of each standard solution was added to the resin and left for 24 h on a rotating plate within an incubator at 37 ± 0.2°C. The tubes were then centrifuged to remove the resin from suspension, and the supernatant was filtered using a 5 -m filter. The equilibrium lactoferrin (CLF*) and lactoperoxidase (CLP*) concentrations of solutions were determined using the bicinchoninic acid protein assay sensitive between 20 and 1200 g/mL. The amounts of protein bound to the resin were calculated from the differences between the initial and final solution protein concentrations and the equilibrium binding capacities for Lf and Lp, QLF* and QLP*, respectively, were calculated by dividing the amounts bound by the volume of the resin.

For column advance studies, Lf concentrations were determined using an optical biosensor analysis as described by Indyk and Filzoni,[16] by means of a surface plasmon resonance technique on a Biacore 3000 instrument. Raw whole milk samples were centrifuged at 4800×g for 2 min to remove fat and filtered using a 5-m filter before serial dilutions (to 2000×) were made in 500 mM HBS-EP buffer (10mM HEPES, pH 7.4 with 3 mM ethylenediamine tetra-acetic acid [EDTA] and 0.005% [v/v] surfactant P20). The administration buffer was obtained from Biacore as 150 mM HBS, and NaCl concentrations were enriched to 500 mM for sample and standard preparations to moderate nonspecific interactions. Lf concentrations were also measured using a bovine Lf ELISA Kit with some changes as described by Turner *et al*.[17]

Lp determinations were carried out using oxidation of synthetic substrate 2,2-azinobis[3-ethylbenzothiazoline-6-sulfonic] diammonium salt for the enzyme. This analyze technique only measures active Lp. Size distributions of resin particles and fat globules were determined by laser light scattering using a Master sizer instrument, samples were first diluted with distilled water to allow sufficient light transmittance. The dv (0.9) (the diameter below which 90% of the volume of particles is found), dv (0.5) (the diameter below which 50% of the volume of particles is found), and dvm (the equivalent volume mean diameter or diameter of spheres of equivalent volume to measured particles) were determined.

By ELISA method

ELISA was carried out with an orientation to the previously published β-lactoglobulin ELISA.[18] Bovine LF antiserum was achieved by vaccinating three rabbits with bovine Lf according to the statement by Harlow and Lane.[19] The bovine Lf was conjugated to the NHS-LC-Biotin (1:5) as described in Mao and Bremel's previous report.[20] In initial tests, the antiserum was diluted 50,000, 100,000, and 200,000 folds in the coating buffer (0.05 M sodium carbonate-bicarbonate; pH 9.6) and coated onto the well of a microplate. The LF-LC-Biotin conjugate was also diluted 100,000, 200,000, and 400,000 folds. Afterward, various amalgamations using the primed antiserum, LF-LC-Biotin, and bovine Lf standard (10 and 0.001 μg/mL) were tested in an ELISA assay for the most optimum titer for antiserum and LF-LC-Biotin. The light absorbance of the above ELISA ranged from 0.1 to 1.5. Finally, the

antiserum and LF-LC-Biotin were established to be the most optimum when diluted 200,000 and 400,000 folds, respectively. Thus, the antiserum was diluted 200,000 fold in coating buffer and dispensed onto an ELISA microplate at 100 µL per well. The microplate was incubated at 4°C overnight and washed 4 times with the washing buffer (0.02 M sodium phosphate, 0.12 M NaCl and 0.025% Tween 20; pH 7.2). Then, 50 µL of assay buffer (0.04 M 3-(N-Morpholino) propanesulfonic acid [MOPS], 0.12 M NaCl, 0.01 M EDTA, 0.5 µg/mL leupeptin, 0.1% gelatin, 0.05% Tween 20, and 0.005% chlorhexidine digluconate; pH 7.2) was added to each well of the plate. The bovine LF standard was prepared with the assay buffer of 10, 1, 0.3, 0.1, 0.03, 0.01, 0.001, and 0 µg/mL, and the milk samples were diluted with the same buffer at 1000–3000-fold. Then, the LF standards or samples in 50-µL analyze buffer were added in triplicate into nonadjacent wells. The Lf -LC-Biotin was diluted 400,000 fold in the assay buffer and 100 µL added into each well. The plate was then sealed and nurtured at room temperature for 2 h. After incubation, the plate was washed 4 times with the washing buffer and blotted on a paper towel. ExtrAvidin peroxidase was diluted 10,000 fold in the assay buffer, and 100 µL was added into each well. The plate was incubated for 2 h at room temperature. After incubation, the plate was washed 8 times with the washing buffer and blotted on a paper towel. The substrate, organized by mixing 19 mL of sodium acetate, 64 µL of hydrogen peroxide was added at 125 µL into each well. After incubating for 12 min, the reaction was stopped by adding 50 µL of 0.5 M H_2SO_4 into each well. The absorbance of each well was read at 450 nm (minus light absorbance at 600 nm as background value) with an auto spectrophotometer. In addition, the intra assay and inter assay coefficients of variances for this ELISA were maintained at below 10%.

Isolation of goat Lf was purified from colostrum.[21] In brief, 60 mL of colostrum (Alpine goat) was defatted by centrifugation at 2000 ×g for 30 min at 4°C. The pH of the skim milk was adjusted to 4.6 with 5 N HCl and then centrifuged at 10,000 ×g for 1 hr to remove the casein precipitate. The whey was passed through a 0.45 mm filter (Millipore, Ireland) to completely remove the casein precipitate and its pH was rearranged to 6.0 with 1N NaOH. The immunoglobulin in the whey was removed by ammonium sulfate precipitation (48%). After passing through 0.45 mm filter, the whey was concentrated and desalted using a desalting kit (Vivascience, UK). The solution in the whey was then replaced with 0.005 M sodium phosphate buffer (pH 6.0) followed by loading into a heparin affinity column (Amersham Pharmacia Biotech, Uppsala, Sweden) and eluted by step elution with 0.005 M sodium phosphate buffer (pH 6.0) containing 0.1, 0.3, or 0.5 M NaCl. The LF was collected at the 0.5 M NaCl eluting solution, and its purity was assessed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and Western blotting.

SIGNIFICANCE

Lf is having the immense biological role that comprises antimicrobial activity, antiparasitic activity, anticancer treatment, and dietary constituent that ratifies growth of gastrointestinal tract (GIT) tract of human infants and newborn nonhuman animals immediately on birth. Lf is well-thought-out to be a fragment of the inborn immune system. At the same time, Lf also takes part in specific immune responses, but in an indirect way.[22] Due to its tactical position on the mucosal surface Lf signifies one of the first defense systems against microbial agents attacking the organism mostly through mucosal tissues. Lf affects the growth and propagation of a variety of infectious agents including both Gram-positive and Gram-negative bacteria, viruses, protozoa, or fungi.[23] Lf acts against parasites in various ways. For example, the infectivity of *Toxoplasma gondii* and *Eimeria stiedae* sporozoites is reduced after their incubation with lactoferricin B. It is thought that lactoferricin ruptures parasitic membrane integrity causing successive changes in communications between the host and parasite.[24] Lf as a dietary element which endorses growth of the GIT of human infants and newborn nonhuman animals rapidly on birth when added to an infant formula or given separately as a dietary supplement thus dropping chronic diarrhea, supporting in the management of short gut syndrome, and evading, at least to some extent, and chronic stubborn diarrhea of the infant. The defensive character of Lf has on plentiful occasions been revealed on chemically induced tumors in laboratory rodents. Lf has even been stated to obstruct the expansion

of experimental metastases in mice proved by the works of Bezault *et al*.[25] Lf is capable to cessation the development of human mammary gland carcinoma cells between the G1 and S stage. Such a negative effect on cell proliferation may be credited to the altered manifestation or activity of regulatory proteins.[26] The Lf -dependent, cytokine-mediated stimulation of the activity of NK cells and lymphocytes CD4+ and CD8+, signifies an important factor in defense against tumor growth. There is an increased number of these cells both in blood and lymphatic tissue after the oral supervision of Lf.

The biological function of the Lp system is chiefly active antimicrobial agent and is used as an antibacterial agent in reducing bacterial microflora in milk and milk products as per works of Reiter and Härnulv.[27] Initiation of the Lp system by addition of hydrogen peroxide and thiocyanate prolongs the shelf life of refrigerated raw milk.[28,29] It is fairly heat resistant and is used as an indicator of over pasteurization of milk. A Lp system is claimed to appropriate for the treatment of gingivitis and paradentosis. Lp has been used in toothpaste or a mouth rinse to reduce oral bacteria and consequently the acid produced by those bacteria. A mixture of Lp, glucose, glucose oxidase (GOD), iodide, and thiocyanate is claimed to be effective in the preservations of cosmetics. Antibody conjugates of GOD and to Lp have been found to effective in killing tumor cells *in vitro*, worked by Stanislawski *et al*.[30] In addition, macrophages exposed to Lp are stimulated to kill cancer cells.[31]

CONCLUSION

Lf has been the focus of intense research of late. Due to its unique antimicrobial, immunomodulatory, and even antineoplastic properties, Lf seems to have great potential in practical medicine. Nevertheless, much research and many experiments still need to be carried out to obtain a better understanding of its activity and interactions and to enable the full and safe utilization of this glycoprotein. Lp is accountable for the inactivation of an extensive range of microorganisms and hence significant in the defense mechanism in human secretions such as saliva, tear fluid, and milk. Lately discovered is the anticancer activity. Extraction of Lf and Lp can be done from raw milk by cation exchange chromatography. Separation and purification technologies can be achieved from whey and bovine milk by batch extraction, chromatographic techniques and with hydrophobic ionic liquids. Polyclonal antibodies were made to purified breast milk Lf and used in an ELISA to estimate plasma concentrations in investigations of innumerable aspects of the inflammatory reaction. One-Step chromatographic method for the industrial isolation and purification of Lf from bovine milk raw material, allowing simultaneously extract at least 90% of the contained Lf, Lp, and free of endotoxins, active against microorganisms using ion exchange sorbent based on hydrophilic, macroporous acrylic resin. The invention allows obtaining highly purified Lf selectively with conservation of its biological (bactericidal) activity, the dairy raw materials used to produce the Lf remains suitable for further processing in the food industry.

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