Purification and Characterization of Intact Lactoferrin Found in the Urine of Human Milk-Fed Preterm Infants

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Little is known about the metabolic fate of ingested lactoferrin in human-milk-fed term or preterm infants. Enzyme-linked immunosorbent assays and sodium dodecyl sulfate/polyacrylamide gradient gel electrophoresis (SDS-PAGE) with immunoblots have demonstrated that the urinary excretion of lactoferrin by preterm infants fed exclusively human milk exceeded that by formula-fed infants. The origin and molecular integrity of the excreted lactoferrin, however, are unclear. We have developed extraction and separation procedures involving a stationary phase of immobilized single-stranded DNA (ssDNA) that allows the efficient (>80%) and rapid isolation of pure, intact lactoferrin from infants' urine. We purified lactoferrin to apparent homogeneity from infants' urine in one step, using the immobilized ssDNA with mobile phases containing up to 6 mol of urea per liter. The purified lactoferrin was evaluated by SDS-PAGE; silver-staining revealed one protein band at 78 kDa; immunoblots confirmed the presence of intact lactoferrin. High-performance affinity chromatography with use of immobilized metal ion (Cu²⁺) suggested the presence of intact, iron-saturated lactoferrin. Subsequent chromatography on high-performance reversed-phase (C₁₈) columns independently verified sample identity and purity.

Additional Keyphrases: protein metabolism · metal-binding proteins · immobilized metal affinity chromatography · protein isolation · DNA

Lactoferrin, the major protein component of human colostrum whey (1), is present in several other biological fluids (2). Reportedly, it has growth-promoting (e.g., 3, 4), bacteriostatic (e.g., 5–8), iron-transporting (9, 10), and even immune-modulating properties (11, 12). However, the biological origin and metabolic utilization of lactoferrin by the immature gastrointestinal system of premature and term infants are poorly characterized.

Infants fed human milk excrete more lactoferrin, lysozyme, total IgA, and secretory IgA in feces than do infants fed artificial formulas (13–15). The origin of these immunological factors has been assumed to be human milk. Indirect evidence, however, suggests that these proteins may originate both from dietary milk intake and de novo synthesis by the infant (16). The presence of putative factors in human milk that induce specific immunological responses has been suggested by results of various other investigations as well (17–19). More recently, several investigators have reported higher amounts of lactoferrin and (or) IgA in the urine of infants fed human milk than in the urine of infants fed artificial formulas (20–23). The origins of these proteins are unknown. To determine the origin and quantify the relative concentrations of both intact lactoferrin and its proteolytic digestion products in the relatively small amounts of urine obtained from infants fed human milk, simple and very efficient lactoferrin purification procedures are required.

Materials and Methods

Collection of preterm infants' urine. The urine (diluted with water during collection) of very-low-birth-weight infants who had received only fortified human milk was pooled, aliquoted, and stored at −70 °C. The urine samples were collected during 96-h balance studies performed when the infants were 2.5 and 5 weeks old. The very-low-birth-weight infants met the following criteria: 28- to 30-weeks of gestation; appropriate growth for gestational age; absence of congenital abnormalities and cardiopulmonary, infectious, or gastrointestinal disorders; and tolerance to complete enteral feedings by the 15th postnatal day. Human milk was fortified daily by adding skim and cream fractions derived from pasteurized, lyophilized, mature milk, obtained from human donors, to fresh milk from each infant's mother.

Efficiency of lactoferrin extraction from infants' urine. We incubated, in triplicate, 1 mL of urine with ¹²⁵I-labeled lactoferrin tracer overnight at 4 °C. NaCl (2 mol/L) was added to aliquots of urine to give a final concentration of 0.5 or 1.0 mol/L. Equal volumes of 50 mmol/L phosphate-buffered (pH 7.4) saline (0.15 mol of NaCl per liter) were added to the control samples. To evaluate the effects of urea on recovery in lactoferrin extraction, we added solid urea to give a final concentration of 3 mol/L. The samples were vortex-mixed, then centrifuged at 14,000 × g for 10 min. The supernatant fluid was removed and the precipitate was evaluated for radioactivity.

Single-stranded DNA-agarose affinity isolation of urinary lactoferrin. Calf thymus single-stranded DNA–agarose (ssDNA-agarose) containing 0.61 mg of DNA per milliliter of gel (lot no. 71101; Bethesda Research Laboratory, Gaithersburg, MD) was packed into a 1.5-cm (i.d.) column to a bed volume of 10 mL. The column was washed with water and then equilibrated with 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineneethanesulfonate (HEPES) buffer, pH 7.0, containing 3 mol of urea per liter. The flow rate was maintained at 30 mL/h. The separation procedure was performed at room temperature (22 to 25 °C). Trace amounts of ¹²⁵I-labeled human lactoferrin (prepared by the lactoperoxidase method of Marchaloniis (24) by use of a kit from Bio-Rad Labs., Richmond, CA) were added to 20 mL of pooled preterm infant urine. After adding urea to give a

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²Nonstandard abbreviations: ssDNA, single-stranded DNA; HEPES, 4-(2-hydroxyethyl)-1-piperazineneethanesulfonate; and SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gradient gel electrophoresis.
Concentration of 3 mol/L and adjusting the pH to 7.0, we centrifuged the urine sample (1000 × g, 10 min), loaded it onto the ssDNA-agarose column, then washed the column with 30 mL of HEPES buffer (pH 8.0, 20 mmol/L) containing 6 mol of urea per liter. Urea was subsequently eliminated by washing with three bed volumes of pH 8.0 HEPES buffer. Nonlactoferrin proteins and lactoferrin emerged after elution with three bed volumes of 0.2 and 0.5 mol/L NaCl, respectively. We collected 2-mL fractions and determined for each its ultraviolet absorbance at 280 nm and radioactivity. To regenerate the ssDNA-agarose column, we washed it with guanidine hydrochloride, 8 mol/L in pH 8 HEPES buffer, followed by water.

Sodium dodecyl sulfate/polyacrylamide gradient gel electrophoresis (SDS-PAGE) of purified lactoferrin. After concentrating the samples from the ssDNA-agarose column 100- to 300-fold in a Centricon-10 (Amicon, Danvers, MA) centrifugal concentrator (M, 10 000 cutoff) and centrifugation, we mixed 50- to 100-μL aliquots with 50 mL of solubilizing buffer (20 g of SDS and 30 mL of mercaptoethanol per liter) and heated these in a boiling water bath for 90 s. Electrophoresis on a 10 to 20 g/100 mL polyacrylamide separating gel with 3 g/100 mL stacking gel was essentially according to the method of Laemmli (25). After electrophoresis, the gel was fixed with acetic acid/water/methanol (10/50/40 by vol), then stained with silver according to the method described by Morrissey (26), with minor modifications. Low-molecular-mass markers (Pharmacia LKB Biotechnology Inc., Piscataway, NJ)—phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), alpha-lactalbumin (14 kDa), and standard human milk lactoferrin (78 kDa; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA)—were used to determine the molecular mass of the purified urinary lactoferrin.

High-performance reversed-phase chromatography. A 50-μL aliquot of the purified lactoferrin sample obtained from ssDNA-agarose was applied to a C_{18} column (30-nm pore diameter, 5-μm particle size; Custom LC, Houston, TX) and eluted with a linear gradient of 35% to 45% solvent B in solvent A over 30 min (solvent A: trifluoroacetic acid, 1 mL/L in water; solvent B: trifluoroacetic acid/acetonitrile/water, 1.95/4.9 by vol). The flow rate was 1 mL/min and protein was detected by ultraviolet absorbance at 220 nm (Beckman System Gold HPLC with a Model 166 or 167 ultraviolet detector; Beckman Instruments, Fullerton, CA).

The concentration of protein purified by affinity chromatography (intact lactoferrin) was estimated by integration of the peak area obtained during analytical reversed-phase HPLC with columns calibrated with known quantities of lactoferrin standards.

High-performance immobilized metal ion (Ca^{2+}) affinity chromatography. An aliquot of concentrated sample from ssDNA-agarose was applied to a TSK gel chelate-5PW (7.5 × 75 mm, 10-μm bead diameter; Tosoh Manufacturing Co., Yamaguchi, Japan) loaded with Ca^{2+}. Preparation of the column and loading conditions were as described previously (27) except that 3 mol of urea per liter was included in all buffers. The column was equilibrated with sodium phosphate buffer (pH 7.5; 20 mmol/L) containing 3 mol of urea and 0.5 mol of NaCl per liter. The sample was eluted with a pH gradient from 7.5 to 4.0 (per liter, 0.1 mol/L of sodium phosphate, 3 mol of urea, and 0.5 mol of NaCl, pH 4.0) at a flow rate of 1 mL/min (Beckman System Gold HPLC). Eluted proteins were detected by their radioactivity and (or) absorbance at 280 nm.

Immunoblot analyses. Western transfer of the electrophoresed samples to nitrocellulose was performed essentially as described by Towbin et al. (28). The nitrocellulose sheet was blocked with gelatin, 30 g/L, then incubated with polyclonal rabbit anti-human lactoferrin antibody (Dako Corporation, Santa Barbara, CA). After washing the nitrocellulose sheet, we incubated it with goat anti-rabbit IgG–horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA). The bound conjugate was made visible by applying 4-chloro-1-naphthol with hydrogen peroxide in methyl alcohol.

Results

Enzyme-linked immunoassays performed as reported earlier (22, 23) indicated that the pool of infants' urine used in these investigations contained approximately 0.1 to 0.3 μg of lactoferrin per milliliter. Optimal extraction of lactoferrin from urine involved minimizing the loss of lactoferrin during sample preparation. Table 1 illustrates the influence of sodium chloride and urea on the extraction efficiency of lactoferrin from the urine of preterm infants. These results confirm previous reports of the tendency of lactoferrin to associate with other proteins and macromolecular structures—in particular, serum albumin and immunoglobulins found in urine of preterm infants (2). Inclusion of 1 mol of sodium chloride per liter or, preferably, for ssDNA affinity chromatography, 3 to 6 mol of urea per liter was useful to inhibit the association of lactoferrin with proteins that are insoluble and are precipitated during centrifugation of the infant urine.

Figure 1 illustrates the use of ssDNA-affinity columns for the one-step purification of intact lactoferrin from the urine of human-milk-fed preterm infants. Lactoferrin labeled with 125I was added (less than 20 ng) as a tracer to the unfractionated urine, but not to those separate purifications of urinary lactoferrin to be evaluated by the immunoblot technique. Analytical recovery of radiolabeled lactoferrin from the immobilized DNA affinity columns routinely exceeded 85%. Optimal recoveries were obtained if the urine was adjusted to pH 7.0 after the addition of the urea (3 to 6 mol/L). Similarly, the highest recovery and purity were achieved when the ssDNA-agarose column was equilibrated with 3 mol/L urea buffer at pH 7.0. Table 2 summarizes the purification data associated with the ssDNA-affinity purification experiment shown in Figure 1. The data shown in Figure 1 are representative of 34 similar purification experiments, for which purification factors of 7000- to 10 000-fold were routinely obtained. Lactoferrin was not detectable in the urine from formula-fed preterm infants, even when 40 mL of urine was processed by ssDNA-affinity chromatography and SDS-PAGE.
proteins were also detectable in the concentrated commercial lactoferrin standard preparation. Although these procedures were not strictly quantitative, the concentration of the lower-molecular-mass proteins detected by the immunoblot analyses appeared to be several orders of magnitude lower than that of purified lactoferrin.

The urinary lactoferrin isolated by ssDNA-agarose affinity chromatography was further characterized for its identity and purity by means of high-performance reversed-phase (C$_{18}$) chromatography, with commercially available human milk lactoferrin used as a control. The purified urinary lactoferrin appeared as a single, sharp peak during elution from the C$_{18}$ reversed-phase column. The human milk lactoferrin used as a control had identical chromatographic characteristics on the reversed-phase column (Figure 4). Figure 5 illustrates the chromatographic behavior of the purified urinary lactoferrin on high-performance columns containing immobilized Cu$^{2+}$ ions. Elution of the purified lactoferrin with a decreasing pH gradient revealed a form of lactoferrin eluted in a manner distinguishable from iron-saturated or hololactoferrin isolated from human colostral whey. We have found the high-performance immobilized Cu$^{2+}$ affinity column to be a sensitive indicator of protein surface architecture and structural homogeneity (31). Thus, co-elution of the purified urinary lactoferrin with lactoferrin purified from human colostral whey may be taken as a good indicator of structural identity.

**Discussion**

In this study, we have shown that the affinity of intact lactoferrin for ssDNA enables lactoferrin to be purified in one step from the urine of human-milk-fed preterm infants.
We have discussed previously the possibilities of promoting the selective adsorption of DNA-binding proteins to immobilized DNA, using urea as a reagent for modifying mobile phases (32). The significance of lactoferrin affinity for DNA is currently under investigation in several laboratories, with reports of high-affinity lactoferrin interactions with DNA, both extracellular and intracellular. The antigens responsible for the generation of autoantibodies in women with systemic lupus erythematosus have been identified as lactoferrin–nucleic acid complexes (33). Bennett et al. (34) have demonstrated that lactoferrin interactions with both double-stranded and single-stranded DNA are of high affinity ($K_d = 6.2 \times 10^{-6}$ mol/L). Lactoferrin interaction with neutrophilic membranes has been shown to occur via membrane-immobilized DNA (35). Finally, different cell types cultured in the presence of lactoferrin can be shown by immunohistochemical means to internalize lactoferrin (36, 37), with intracellular perinuclear localization.

We have not investigated the specificity of lactoferrin for DNA. Some have suggested that lactoferrin interacts with DNA in a sequence-specific manner (34, 35). We have found the interaction of lactoferrin with immobilized ssDNA-agarose to be of moderate to high affinity ($K_d = 1 \times 10^{-5}$ mol/L). The inability of phosphocellulose to produce the same results (i.e., selective adsorption of lactoferrin) suggests that the immobilized ssDNA is selectively interacting with lactoferrin via a combination of mechanisms, such as electrostatic and nonelectrostatic (e.g., hydrophobic) interactions.

In separate experiments in which lactoferrin was purified to homogeneity from human colostral whey, frontal analysis chromatography showed that the capacity of ssDNA-agarose for lactoferrin, under the conditions used for purification of the urinary lactoferrin (3 to 6 mol of urea per liter), exceeded 23 mg/mL. Although we have routinely used 10-mL columns for the results presented here, it is possible to use relatively small columns (1-mL bed volume) of the ssDNA-agarose to extract the lactoferrin from 10 to 20 mL of diluted infants' urine. The columns are, therefore, "Disposable" and the maintenance of low background contaminations is ensured. Lack of extensive equipment requirements is another advantage of the procedure to purify urinary lactoferrin we have described. The lactoferrin is adsorbed and extracted from the DNA column in batches. Several columns may be processed simultaneously by using multiport vacuum manifold equipment (J. T. Baker, Phillipsburg, NJ).

The physiological significance and origin of the intact lactoferrin in the urine of these infants remain under investigation, particularly given the recent reports of enhanced urinary excretion of lactoferrin in premature infants fed human milk (21–23). The immunological techniques used in these reports, however, do not distinguish the quantitative contributions of the intact lactoferrin from those of the lactoferrin fragments also present in the urine.

The application of this method to the purification of lactoferrin from other secretions and biological fluids (e.g., tears, saliva, serum, vaginal secretions, seminal fluid, or synovial fluid) promises to facilitate the investigation of lactoferrin for its structural variations and metabolic processing.
The fate of dietary lactoferrin may be traced by collecting milk from mothers who have received amino acids labeled with stable isotopes. The lactoferrin from this milk may then be purified to homogeneity and its isotopic enrichment evaluated by mass spectrometry. We can feed these milk preparations to preterm and term infants and purify the lactoferrin from their tears, saliva, urine, or feces, on the basis of the procedure outlined here, then evaluate the stable isotopic enrichment of these biological products. Such investigations are in progress and will facilitate the determination of the origin of lactoferrin in these secretions.

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