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Interaction of Human Lactoferrin with DNA: One-Step Purification by Affinity Chromatography on Single-Stranded DNA-Agarose[1,2]

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ABSTRACT. Human lactoferrin has been purified to apparent homogeneity with high recovery (>95%) in one chromatographic step using immobilized single-stranded DNA-agarose. Human colostral whey, at neutral pH, was loaded onto columns of DNA-agarose; a single purified protein, lactoferrin, was eluted using elevated ionic strength buffers. The lactoferrin purified in this manner was shown to be homogeneous by high-performance ion-exchange chromatography (Mono-S), immobilized metal ion (Cu^{2+}) affinity chromatography, and reverse-phase (C_{18}) chromatography. Electrophoresis on SDS-polyacrylamide gradient (10–20%) gels and silver staining showed the purified lactoferrin preparation to contain a single protein of 78 kD with intact immunologic determinants. Similar results were obtained before and after iron saturation of the colostral whey proteins. Apolactoferrin purified in this manner was shown to bind iron with high affinity. These results demonstrate the effectiveness of immobilized DNA as one of the most rapid and complete lactoferrin purification procedures described thus far. (*Pediatr Res* 26: 618–622, 1989)

Lactoferrin is a ubiquitous, metal-binding, secretory protein (1–3) known to play a role in host defense (2, 4), iron and trace element metabolism (1, 4–9), and cell proliferation (10–12). It is the major protein found in human colostral whey (13), and is found in the milk of several other species (1). Although the structure of human lactoferrin is known (2, 3, 14), the full biologic significance of this protein, its mechanism of action, and diagnostic potential remain the subject of intense investigation. Thus, the need is great for simple and rapid means of lactoferrin purification at both the analytical and preparative scale levels.

Several previous investigations (1) and our own experience have shown that lactoferrin tends to become tightly associated with other macromolecules, including DNA (15–18). These adsorption properties, however, have never been exploited for the purification of lactoferrin. We have found immobilized single-stranded DNA to be effective for the purification of steroid receptor proteins, especially in the presence of urea as a mobile phase modifier to prevent the adsorption of other nonspecific DNA-binding proteins (19). We report here that immobilized DNA can be used effectively for the rapid and complete purification of human lactoferrin.

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MATERIALS AND METHODS

Colostrum from volunteer mothers was provided by the Children's Nutrition Research Center Human Milk Bank, Department of Pediatrics, Baylor College of Medicine. The colostrum was collected from each breast with a mechanical pump on d 2 to 4 postpartum and stored at −80°C until use.

Whey preparation and iron saturation. Frozen colostrum was thawed at 37°C, adjusted to pH 4.3, and incubated for 1 h at 4°C. Casein and fat were removed by centrifugation at 45 000 rpm for 1 h at 4°C in an SW 60 rotor (Beckman Instruments, Inc., Palo Alto, CA). After centrifugation, the fatty layer was removed and the whey proteins decanted. The whey was filtered (0.8 μm) and readjusted to pH 7 or 8 before fractionation. For those preparations of whey to be labeled or saturated with iron, the whey (5 mL) was brought to 0.1 M $NaHCO_3$, and approximately 100 000 cpm of ^{59}Fe was added to the sample before incubation at 37°C for 10 min. Finally, $FeSO_4$ was added to the sample and incubation was resumed for another 10 min. The pH was then adjusted to pH 8.0 before purification of the lactoferrin by affinity chromatography on single-stranded DNA-agarose.

Affinity chromatography on single-stranded DNA-agarose. Single-stranded DNA-agarose (lot 71101; 0.61 mg DNA/mL gel) was obtained from Bethesda Research Laboratory (Gaithersburg, MD) and packed into a 1.5 cm i.d. column to a bed volume of 5 mL. The column was washed with water and was equilibrated with 20 mM HEPES buffer (pH 8.0), plus or minus 6 M urea, at a flow rate of 30 mL/h. The separation procedure was performed at room temperature (20–25°C). Where noted, solid urea was added to those whey samples (to 6 M) in which lactoferrin was purified in the presence of urea. A trace amount of ^{125}I-labeled human lactoferrin (Jackson Immunoresearch Laboratory, Westgrove, PA) was added to the samples of whey (5 mL) before addition to the DNA affinity column. The columns were washed (10 × bed volume) with column equilibration buffer. Before gradient elution, the urea (when present) was removed with several column volumes of 20 mM HEPES buffer (pH 8.0). Lactoferrin was eluted with a linear gradient of NaCl (0–1.0 M) in 20 mM HEPES pH 8.0 buffer. Fractions of 1 mL each were collected. Absorbance at 280 nm and radioactivity were measured in each of the collected fractions. After each purification procedure, the DNA-agarose column was washed extensively

with 8 M guanidine-HCl in 20 mM HEPES buffer at pH 8.0 followed by water.

Chromatographic analyses of purified lactoferrin. High-performance cation-exchange chromatography: Purified lactoferrin obtained by DNA-agarose affinity chromatography was applied to a Pharmacia Mono-S column (0.5 cm i.d. × 5 cm) equilibrated with 50 mM Tris-HCl, pH 8.0. After sample addition, elution was initiated using a linear NaCl gradient (0–1 M) in 50 mM Tris-HCl (pH 8.0) at a flow rate of 1.0 mL/min. Protein elution was monitored by UV absorbance at 280 nm.

High-performance immobilized metal ion affinity chromatography: An aliquot of purified lactoferrin sample from the DNA-agarose column (see Fig. 1*B*: peak 1) was applied to a TSK gel chelate-5PW (7.5 × 75 mm, 10 μm bead diameter, ToSoh Manufacturing Co., Sinnanyo-shi, Japan) loaded with Cu(II). Preparation of the column and loading conditions were exactly as described previously (20). The column was equilibrated with 20 mM sodium phosphate buffer (pH 7.5) containing 3 M urea and 0.5 M NaCl. The sample was eluted with a pH gradient from 7.5 to 4.0 (0.1 M sodium phosphate, 3 M urea, 0.5 M NaCl, pH 4.0) at a flow rate of 1 mL/min. Protein elution was monitored by determining UV absorbance (280 nm).

High-performance reverse-phase chromatography: An aliquot (250 μL) of purified lactoferrin collected from the DNA-agarose affinity column was applied to a C_{18} reverse-phase column (7.5 mm inner diameter × 25 cm; 300 Å pore size, 5 μm particle diameter, Customsphere, Houston, TX). Elution was achieved by a linear gradient of 30 to 45% solvent B in solvent A over 30 min (solvent A: 0.1% trifluoroacetic acid in water, solvent B: 95% acetonitrile in 0.1% trifluoroacetic acid) at a flow rate of 1 mL/min. Protein elution was monitored by UV absorbance (220 nm).

SDS-PAGE. An aliquot of the isolated lactoferrin sample was mixed with an equal volume of solubilizing buffer (containing 2% SDS, 3% mercaptoethanol), heated in a boiling water bath for 90 s, and electrophoresed on a polyacrylamide gradient gel with stacking gel essentially according to the method of Laemmli (21). If required, the sample of purified lactoferrin collected from the DNA-agarose affinity column was concentrated using a Centricon (Amicon, Danvers, MA) concentrator (10 000 mol wt exclusion). After electrophoresis, the gel was fixed in a 10% acetic acid solution with 50% methanol. The gel was silver-stained according to the method of Morrissey (22).

Iron-binding analyses of purified lactoferrin. The UV/VIS absorption spectrum (600–190 nm) of purified, iron-saturated lactoferrin was determined in 20 mM HEPES buffer at pH 8.0 on a Beckman model DU-70 spectrophotometer. The ratio of absorbances at 465 and 280 nm was used to evaluate the degree of iron saturation. The ^{59}Fe-binding capacity of the purified lactoferrin was evaluated as described above. The sample was adjusted to 0.1 M $NaHCO_3$, and approximately 100 000 to 200 000 cpm of ^{59}Fe was added before incubation at 37°C for 10 min. On the basis of the concentration (determined by absorption spectroscopy) and volume of lactoferrin in the ^{59}Fe-labeled sample, an appropriate amount of $FeSO_4$ was added, and incubation was resumed for another 10 min. Lactoferrin-bound ^{59}Fe in the sample was determined by chromatography on high-performance size-exclusion columns (TSK-3000SW) or Mono-S ion-exchange columns.

Immunochemical evaluation of purified lactoferrin. Ouchterlony (23) immunodiffusion analyses of the purified lactoferrin preparations were performed using polyclonal antihuman lactoferrin antisera obtained from Dako Laboratories (Santa Barbara, CA). Immunoblots of SDS-PAGE were performed as outlined previously (24) using a peroxidase-conjugated second antibody.

RESULTS

The gradient elution profiles shown in Figure 1*A* and *B* demonstrate the chromatographic behavior of human colostral whey proteins and lactoferrin on single-stranded DNA. ^{125}I-labeled lactoferrin was added to the whey to monitor lactoferrin elution characteristics and to estimate recovery. The single major protein elution peak (1) in each of the experiments coeluted with the trace amounts of ^{125}I lactoferrin or ^{59}Fe. Purification of lactoferrin from the whey preparations brought to 6 M urea had

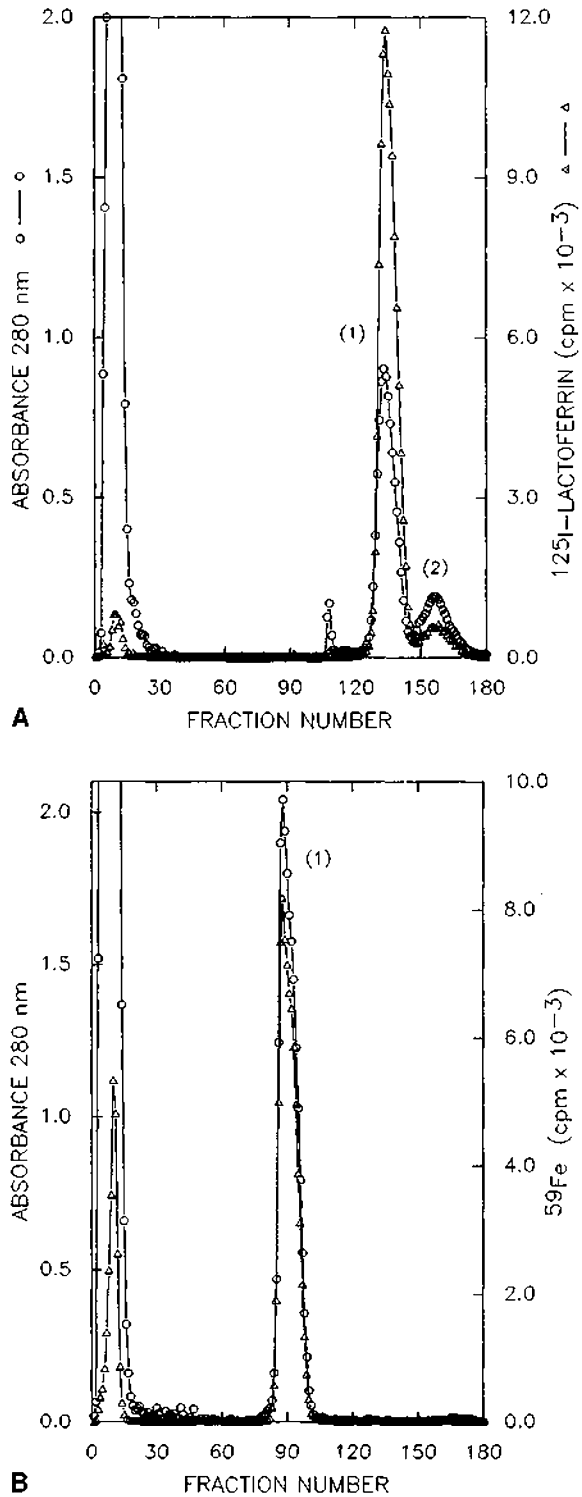

Fig. 1. Purification of human milk lactoferrin by affinity chromatography on immobilized single-stranded DNA. ^{125}I-labeled lactoferrin (*A*) or ^{59}Fe (*B*) was added in tracer amounts to determine the elution position of iron-binding proteins and recovery of lactoferrin from the samples of human colostral whey analyzed before (*A*) and after (*B*) iron saturation. Elution gradients began at fractions 110 (*A*) and 60 (*B*).