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Short Communication

High-performance liquid chromatographic separation of human apolactoferrin and monoferric and diferric lactoferrins

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ABSTRACT

A simple chromatographic separation of the different molecular forms of human milk lactoferrin according to their iron contents is described. The appropriate conditions were developed with a Mono-S cation-exchange column linked to an automated Urtrochrom GTi system. The pure lactoferrin with different iron binding (apolactoferrin, monoferric lactoferrin and diferric lactoferrin) separated into three peaks. The three peaks were eluted from the Mono-S column with 0.88, 0.97 and 1.05 *M* sodium chloride in 10 m*M* phosphate buffer (pH 7.0).

INTRODUCTION

Lactoferrin is an iron-binding protein found in many external secretions [1]. In mammals, the concentration of lactoferrin in milk depends on the stage of lactation [2] and the species. Human milk contains between 1 and 2 mg/ml [3], whereas bovine milk contains only 0.1 mg/ml [2]. Lactoferrin consists of a single polypeptide chain of relative molecular mass *ca.* 80 000, to which two glycans are attached through N-glycosidic linkages [4]. The two N-acetyllactosaminic-type glycans are structurally heterogeneous [5] and differ from those of other transferrins [6]. Like all transferrins, lactoferrin possesses two metal-binding sites, each of which can bind a ferric ion (Fe^{3+}) together with a bicarbonate anion. Accordingly, lactoferrin exists in four molecular forms in nature, as does transferrin.

We report here the separation of four molecular forms of transferrin by high-performance liquid chromatography (HPLC) [7]. Our aim was to develop a new direct method for the separation of different molecular forms of human lactoferrin using HPLC.

EXPERIMENTAL

Materials

Materials were purchased from the following suppliers: Mono-S HR 5/5 ion-exchange HPLC

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column from Pharmacia (Uppsala, Sweden); TSK G 2000 gel permeation column from LKB (Bromma, Sweden); CNBr-activated Sepharose 4B from Pharmacia LKB Biochemistry (Uppsala, Sweden); bovine β-lactoglobulin from ICN Biochemicals (Cleveland, OH, USA); desferrioxamine (deferoxamine) from Nihon Ciba Geigy (Hyougo, Japan); anti human lactoferrin from Cooper Biomedical (Cochranville, PA, USA). Human Milk was obtained from healthy donors. Water for HPLC analysis was doubly distilled in glass.

Purification of human lactoferrin

Lactoferrin was purified from human whey using β-lactoglobulin as a one-step affinity purification method [8]. Pooled human milk was thawed at room temperature and the cream removed by centrifugation at 5000 *g* for 30 min at 4°C. All procedures were carried out at 4°C except where noted. The resultant skim milk was adjusted to pH 4.7 with 1 *M* acetate and stirring at 4°C for 16 h to precipitate, predominantly, the casein. After centrifugation as above, the whey was decanted [9].

Human whey was dialysed overnight against 0.1 *M* sodium chloride–10 m*M* potassium phosphate buffer (pH 7.2), and applied at a flow-rate of 7 ml/h to a column containing 8 ml of β-lactoglobulin-Sepharose (2.9 mg of protein per milliliter of settled gel) equilibrated in the same buffer. The column was washed, and the bound protein was eluted with 0.3 *M* sodium chloride. The absorption was monitored at 280 nm, and the lactoferrin concentration was measured by radial immunodiffusion [10].

Preparation of apolactoferrin and diferric lactoferrin

Apolactoferrin (iron-free lactoferrin) was prepared by incubating a mixture containing 500 μg/ml lactoferrin and 0.25 *M* deferoxamine in phosphate-buffered saline (PBS) at 37°C for 1 h. Deferoxamine was removed by dialysis at 4°C against PBS [11].

To produce iron-saturated lactoferrin (diferric lactoferrin), sodium bicarbonate was added at a concentration of 0.1 *M*, then ferrous sulfate in 10 m*M* hydrochloric acid was added before incubation at 37°C for 15 min or longer [12].

Cation-exchange column chromatography

A Mono-S HR 5/5 column was used in the LKB HPLC system (Urtrochrom GTi), consisting of a 2154 HPLC injector, a 2152 LC controller, a 2150 HPLC pump, a 2212 Helirac, a 2210 recorder and a 2151 variable-wavelength detector set at 280 nm. The inlet side of the column was fitted with additional tubing to act as a pre-column. The column was equilibrated at 0.3 ml/min with 10 m*M* sodium phosphate buffer (pH 7.0), which was degassed and filtered through a 0.22-μm membrane filter (type Sterivex-GV, Millipore). The sample, which was dialysed against the above buffer in volumes up to 3 ml, was then loaded through the injection valve into a Superloop and introduced into the column. After sample application, the column was washed with the above buffer for 5 min. Lactoferrin adsorbed on the column was eluted with a linear salt gradient from 0 to 1.5 *M* sodium chloride in 10 m*M* sodium phosphate buffer (pH 7.0), after which the column was washed for 10 min with 2.0 *M* sodium chloride in the above buffer prior to a return to the initial conditions for the next sample.

RESULTS AND DISCUSSION

Lactoferrin was purified from human pooled milk by affinity chromatography under the conditions described in Experimental. The purity of the purified lactoferrin was checked by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1) and immunoelectrophoresis (not shown), and it was then used as the starting material for all the subsequent purification procedures (not shown). The HPLC profiles of the pure lactoferrin with different degrees of iron binding are shown in Fig. 2. Fig. 2a illustrates a typical separation of a sample of 400 μg of purified lactoferrin with a 10 m*M* phosphate buffer system. The sample contained apolactoferrin, monoferric lactoferrin and diferric lactoferrin. The three peaks from the Mono-S column

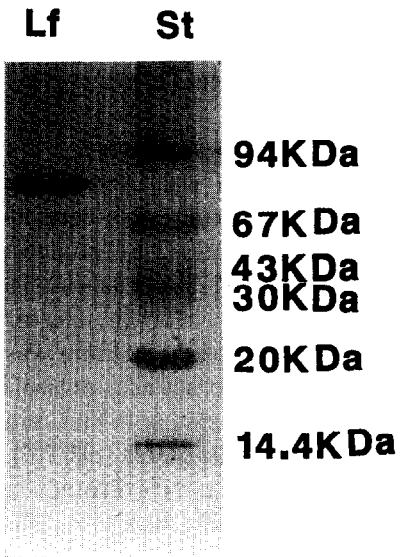

Fig. 1. SDS-PAGE of purified lactoferrin. Lf = purified lactoferrin; St = molecular mass markers. KDa = kilodalton.

corresponded to 0.88, 0.97 and 1.05 *M* sodium chloride in 10 m*M* phosphate buffer (pH 7.0). To verify the purity of these three peaks, the individual fractions were separated under the above HPLC conditions. These fractions (A, B and C) were homogeneous and maintained identical retention volumes (sodium chloride concentration: 0.88, 0.97 and 1.05 *M*) when rechromatographed on the same Mono-S column (data not shown). The slight variation of the pH of the elution buffer affected the retention times, but the HPLC profiles remained unchanged.

In the purified lactoferrin tested, the sum of fractions B and C ranged about 95% of a gross sample (Fig. 2a). However, in such a case, if lactoferrin was saturated with ferric ions in the purification step, the sum of fractions A and B ranged about 95% (data not shown). The samples shown in Fig. 2b and c correspond respectively to diferric lactoferrin and apolactoferrin, prepared as described in Experimental.

These results clearly demonstrate that diferric and monoferric lactoferrin and apolactoferrin in purified human lactoferrin were eluted with 0.88, 0.97 and 1.05 *M* sodium chloride in 10 m*M* phosphate buffer (pH 7.0). The HPLC method de-

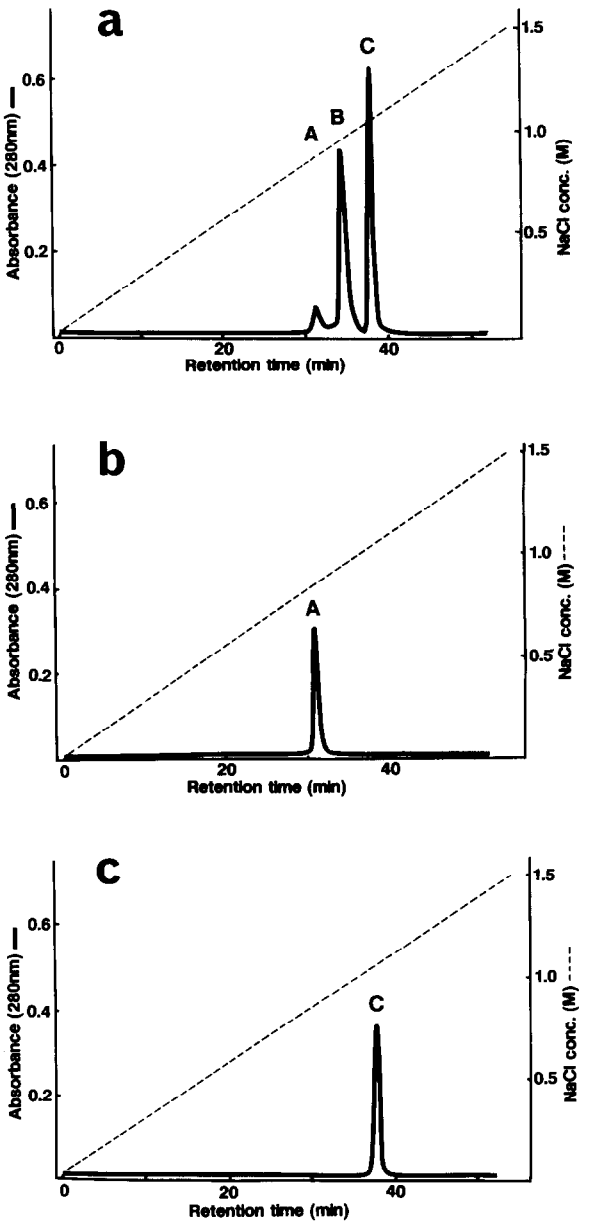

Fig. 2. Ion-exchange chromatographic separation of lactoferrin on a Mono-S HR 5/5 column (5 × 0.5 cm I.D., 10 μm) using a linear gradient of sodium chloride (0–1.5 *M*) in 10 m*M* phosphate buffer (pH 7.0): (a) purified lactoferrin; (b) diferric lactoferrin; (c) apolactoferrin. Flow-rate, 0.5 ml/min; detection wavelength, 280 nm; applied sample volume, (a) 500 μg, (b) 100 μg, (c) 100 μg. Peaks: A = diferric lactoferrin; B = monoferric lactoferrin; C = apolactoferrin.

scribed here allows the separation of monoferric lactoferrin under non-denaturing conditions from a mixture of lactoferrins with various degrees of iron content. N-Site and C-site iron-binding lactoferrins of the two monoferric lactoferrin species were not distinguished under our experimental conditions. The analytical Mono-Q HR anion-exchange column did not separate individual lactoferrins.

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