Preparation of Lactoferrin by Hydrophobic Interaction Chromatography from Milk Acid Whey

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ABSTRACT
Lactoferrin was isolated from 1.8 M ammonium sulfate whey, which had been prepared from bovine milk acid whey, by hydrophobic interaction chromatography using Butyl Toyopearl 650M and by DEAE ion exchange chromatography. The hydrophobic interaction chromatography of 1.8 M ammonium sulfate whey supernatant resulted in concentration of the whey proteins and fractionation into a fraction eluting in deionized water, a fraction eluting in .25 M acetic acid, and a fraction eluting in .2 N NaOH. Lactoferrin was contained only in the fraction released by .25 M acetic acid from the matrix of Butyl Toyopearl 650M. It is estimated that approximately 80 mg of lactoferrin can be obtained by this method from 1 L of neutralized acid whey. The isoelectric point of lactoferrin is between pH 4.8 and pH 5.3 as determined by isoelectric focusing.

INTRODUCTION
Sørensen and Sørensen (15) first noted the existence of a red protein in bovine milk whey, and Polis and Shmukler (12) later isolated it in partially purified form during the preparation of lactoperoxidase. The red protein was isolated from the acid casein fraction by extraction with 1 M acetic acid at pH 4 (6) and from the acid whey fraction by Groves (7). Blanc and Isliker also isolated lactoferrin from the centrifugal supernatant of bovine skim milk (3). This red protein was called lactoferrin and showed only a broad absorption maximum around 470 nm, but no Soret band was found (3, 6, 18). A molecular weight of 86,100 was calculated for lactoferrin (4). In the same period Gordon et al. (5) isolated a similar, but colorless, protein from rennet whey. Usually lactoferrin can be concentrated by an ammonium sulfate precipitation of milk albumin fraction and is further purified by DEAE-cellulose column chromatography (6). However, lactoferrin is not easily separated and purified from bovine milk acid whey because lactoferrin content in milk is very small compared with other bovine milk albumin contents.

In the present paper, lactoferrin was prepared from milk acid whey. When ammonium sulfate concentration was elevated to 1.8 M, most globulins were precipitated (15). Proteins in the supernatant from ammonium sulfate whey were adsorbed to the matrix for hydrophobic interaction chromatography. Most of the whey proteins, such as α-lactalbumin and β-lactoglobulin, were released from the matrix by decreasing salt concentration; however, a part of lactoperoxidase and lactoferrin still remained in the matrix. The remaining two colored proteins were released by acetic acid and then were separated by DEAE ion exchange chromatography. Characteristics of lactoferrin result in strong adsorption to the matrix by hydrophobic interaction chromatography (21) and release from the matrix by .25 M acetic acid.

MATERIALS AND METHODS
Ammonium Sulfate Whey from Milk

Skim milk was prepared by centrifugation at 2000 × g for 20 min at 30 to 40°C from pooled a.m. milk from Holstein cows. Skim milk was acidified to pH 4.6 at 20°C by addition of 2 N HCl and casein was removed by centrifugation at 2000 × g for 20 min at room temperature.
Acid whey was neutralized to pH 6.8 by addition of 2 N NaOH. An amount of 801 g ammonium sulfate was added to the 3 L of neutralized acid whey and allowed to stand for 3 h at room temperature. Precipitated globulins were removed by centrifugation at 2000 × g for 20 min and by filtration using Toyo filter paper No. 2 (Toyo Roshi, Tokyo, Jpn.). A volume of 3300 ml of 1.8 M ammonium sulfate whey was obtained.

Hydrophobic Interaction Chromatography

Butyl Toyopearl 650M (BTP-650M) (Toyosoda, Tokyo, Jpn) was prepared for hydrophobic interaction chromatography (14, 21, 22). The 1.8 M ammonium sulfate whey supernatant was passed through an 800-ml matrix, 5-cm × 40-cm column (BTP-650M in equilibrium with 1.8 M ammonium sulfate), and this matrix was washed with 800 ml of 1.8 M ammonium sulfate solution. All proteins in the 1.8 M ammonium sulfate whey were adsorbed to the matrix of BTP-650M, because no protein was detected from the solution that was flow out from the column. The adsorbed proteins were fractionated with 1200 ml of deionized water, 800 ml of .25 N acetic acid, and 800 ml of .2 N NaOH. The adsorbed proteins were fractionated into three fractions; a fraction eluting in deionized water (DW); a fraction eluting with acetic acid (AA); and a fraction eluting with NaOH (Na). The DW and Na fractions were prepared for further experiments for the isolation of other proteins. The AA fraction was neutralized to pH 6.8 by addition of .2 N NaOH.

Separation of Albumin and Globulin

Globulins and albumins were separated by adding 28 g of anhydrous sodium sulfate into 140 ml of AA fraction at 40°C according to Aschaffenburg and Drewry (2). The precipitate of globulin was removed by centrifugation at 20,000 × g for 30 min at room temperature. The supernatant, albumin from AA fraction, was dialyzed against deionized water for 2 d in a cold room to remove sodium sulfate.

Diethylaminoethyl Ion Exchange Chromatography

Diethylaminoethyl Toyopearl (DEAE-TP) (Toyosoda, Tokyo, Jpn.) was prepared for ion exchange chromatography. The DEAE-TP chromatography was made in a 1.5-cm × 18-cm column with 200 ml of pH 8.5, .05 M Tris-HCl buffer and 200 ml of same buffer containing .3 M NaCl linear gradient, which was performed as described previously (21).

RESULTS AND DISCUSSION

During an investigation of the chromatographic preparation of minor whey proteins, lactoferrin was isolated through hydrophobic
interaction chromatography using BTP-650M and was purified by DEAE ion exchange chromatography. The procedure of hydrophobic interaction chromatography has two results: 1) concentration of whey proteins and 2) fractionation of the protein into DW, AA, and Na fractions. Lactoferrin was found in the AA fraction only.

The result is shown in Figure 1. The first (DW) fraction was eluted by the decreasing of hydrophobic interaction between proteins and the matrix using deionized water. This fraction contained the main proteins of the acid whey such as β-lactoglobulin, α-lactalbumin, and other minor proteins, including a part of lactoperoxidase. The second fraction (AA) was eluted by lowering the pH, and it contained the remaining lactoperoxidase and all the lactoferrin. The DW and AA fractions showed a yellow color, and their maximum absorption was at 412 nm, which is the characteristic Soret band of lactoperoxidase (13, 22). The dotted lines in the DW and AA fractions in Figure 1 show the existence of lactoperoxidase. However, the third fraction (Na) also appeared clear light yellow with an absorption maximum at 425 nm. Most likely this is due to the presence of lumichrome at alkaline pH (9). The Na fraction contained unidentified materials that strongly bound to the matrix. However, no lactoferrin was detected in the DW fraction or the Na fraction. It was isolated only in the AA fraction. As for lactoferrin, 3 L of acid whey were concentrated to 140 ml of AA fraction.

Figure 1. Elution pattern for hydrophobic interaction chromatography of 1.8 M ammonium sulfate whey (3300 ml) from a BTP-650M column (5 cm × 40 cm). Eluant: deionized water (1200 ml), 0.25 M acetic acid (800 ml), and 0.2 M NaOH (800 ml). Solid line, absorbancy at 280 nm; dotted line, ○ absorbancy at 412 nm for DW fraction and AA fraction, Δ absorbancy at 425 nm for Na fraction.

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After the globulins had been removed from the AA fraction by Aschaffenburg and Drewry’s method (2), the remaining albumin was 301 mg. The DEAE ion exchange chromatography was then carried out using DEAE-TP for separation of lactoferrin from albumin of the AA fraction. The result is shown in Figure 2. Two peaks were fractionated. The protein in the first peak had a light yellow color with a large absorption peak at 412 nm, which corresponded to the Soret band. Consequently, it could be identified as lactoperoxidase. The protein in the second peak was red with a broad absorption maximum at 470 nm, which agreed with the absorption curve of lactoferrin (3, 6, 18). This also agreed with Groves’ results (7) that the red protein, lactoferrin, was eluted at higher salt concentration and pH than was lactoperoxidase. A single band was observed for this fraction upon SDS-PAGE, and its molecular weight was estimated at 87,000 dal as shown in Figure 3. Albumins in the AA fraction contained 301 mg of protein, which were separated from 3 L of acid whey and 150 mg of this albumin was purified by DEAE-TP. After separation by DEAE-TP chromatography, lactoferrin content was estimated to be approximately 80% of the albumin in the AA fraction, i.e., 80 mg/L milk acid whey.

The isoelectric point was estimated to be between pH 4.8 to 5.3 by isoelectric gel electrophoresis. The isoelectric point for lactoferrin was reported to be pH 8.0 ± .2 by Szuchet-Drechin and Johnson (18) and pH 7.8 by Groves (6); however, these values did not agree with the results of our isoelectric focusing disc gel electrophoresis.

The red protein showed no antigenic relationship to bovine transferrin and to bovine whole serum using rabbit antisera by immunoelectrophoretic analysis. From this result, the bovine milk red protein isolated in our experiment was not transferrin but was identified as lactoferrin in agreement with Szuchet-Drechin and Johnson (17) and AL-Mashikhi et al. (1).

Figure 2. Elution pattern for DEAE ion exchange chromatography of the AA fraction of the albumin fraction (100 mg protein) from a DEAE-TP column (1.5 cm × 18 cm). Eluant: 200 ml of pH 8.5, .05 M Tris-HCl buffer and 200 ml of pH 8.5, .05 M Tris-HCl buffer containing .3 M NaCl.
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REFERENCES


