ABSTRACT

Immunoaffinity columns made with monoclonal antibodies to either human or bovine lactoferrins were prepared to isolate human lactoferrin or bovine lactoferrin from milks by a single chromatographic step. Recoveries of human lactoferrin and bovine lactoferrin were 98 and 97%, respectively. The human lactoferrin recovered from defatted human colostrum was 98% pure with 93% iron-binding capacity. Amount of recovered bovine lactoferrin, as well as purity and iron-binding capacity, varied widely depending on the source of bovine milks and pretreatments (particularly pasteurization temperature). The best source to isolate bovine lactoferrin was raw skim milk yielding a protein 97% pure and with a 99% iron-binding capacity. Thus, immunoaffinity chromatography provides an effective one-pass isolation of highly pure human or bovine lactoferrin with reasonable recovery and iron-binding capacity.

INTRODUCTION

Lactoferrin (LF) is an iron-binding glycoprotein found in externally secreted fluids such as milk (8, 24). It is likely that the chelating capability is responsible for its many biological functions. These include a bacteriostatic effect (7, 20, 25, 27), growth promoting effect on lymphocytes (10), and product regulation of macrophage, granulocyte, and neutrophil leukocytes (1, 5, 6, 12). These important functions make LF a potential source of dietetic foods and pharmaceuticals.

A number of methods have been proposed for isolation of LF from milk and subsequent purification. In general, isolation of lactoferrin is performed by chromatography on DEAE-cellulose (9) and CM-Sephadex C-50 (13). Silica particles (23) are alternatively used in place of the ion-exchange resins. Although additional purification steps such as gel filtration and copper affinity chromatography (15) are required to increase purity, these steps often reduce yield and are time-consuming. Blackberg and Hernell (4) reported a one-step isolation of highly pure LF from human milk whey by heparin-Sepharose chromatography with a linear gradient of NaCl (after dialyzing the whey against 0.05 M NaCl in 5 mM veronal-HCl (pH 7.4)). Their method is simple and thus can scale up easily.

An alternative strategy to isolate highly pure LF with reasonable recovery is to employ the highly specific interaction between antigen and antibody. Since Köhler and Milstein (17) have developed cell fusion techniques, one of the important applications of monoclonal antibodies (McAb) is in the isolation and purification of minor components of biological tissues. Staehelin et al. (26) have purified human leukocyte interferon with McAb against leukocyte interferon. The specific activity increased 1150 times with 95% recovery. Thus, it may be beneficial to establish hybridoma cells producing McAb against LF and to prepare an affinity column with the McAb to isolate LF from milks.

This paper describes the isolation of LF from milks by a single passage through an affinity column with immobilized LF-McAb and demonstrates that highly pure LF can be recovered from raw skim milk with a reasonable yield and iron-binding capacity.

MATERIALS AND METHODS

Lactoferrin

Human lactoferrin (HLF) (98% pure) was purchased from Sigma Chemical Co. (St. Louis, MO). Bovine lactoferrin (BLF) for immuniza-
tion was prepared by ion exchange chromatography according to Johansson (13). The purity of the resulting BLF was approximately 70%.

Milk

Human colostrum was kindly supplied from seven healthy volunteers. Raw skim milk was purchased from Zenraku Milk Co., Sayama, Jpn. Cheese whey was supplied by Cheese Institute, Snow Brand Milk Products Co., Ltd., Kobuchizawa, Jpn. The whey had been pasteurized at 75°C for 20 s prior to cheese making. Each specimen was centrifuged at 10,000 × g for 20 min at 4°C; the supernatant was filtered with Toyo #2 (Jpn) filter paper to remove fat and stored at 4°C until used. Skim milk and cheese whey were pasteurized at 65°C for 30 min.

Immunization

A 0.2% solution of HLF or BLF dissolved in 0.01 M phosphate buffer (pH 7.2) containing 0.15 M NaCl [phosphate-buffered saline (PBS)] was mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI) and injected into the abdominal cavities of BALB/c mice aged 6 to 8 wk. Mice were immunized three times at 2-wk intervals.

Cell Fusion

On the d 5 to 7 after the final immunization, spleen lymphocytes were extracted and subjected to cell fusion following the modified method of Oi and Herzenberg (21). Briefly, approximately 2 × 10^8 of spleen lymphocytes and 6 × 10^8 of SP2/0-Ag14 mouse myeloma cells (Flow Laboratories Inc., McLean, VA) suspended in Dulbecco's modified Eagle medium (DMEM; Gibco Laboratories Co., Grand Island, NY) were mixed in the presence of 50% polyethylene glycol (PEG; gas chromatographic grade, M W 4000, Merck Co., Inc., Darmstadt, FRG). The cells were diluted to 1 × 10^7 cells/ml with HT medium: i.e., DMEM containing 10% fetal calf serum (Lot #29101709, Flow Laboratories Inc., North Ryde, Aust), 100 μM hypoxanthine (Sigma), and 16 μM thymidine (Sigma). A 0.2-ml portion of this dilution was seeded in 96 well microtiter plates (Corning Glass Works, Corning, NY). After overnight culture at 37°C under 5% CO₂, half of the HT medium in each well was replaced with fresh HAT medium (HT medium containing 4 μM aminopterin, Sigma, St. Louis, MO) to select hybridoma cells.

Screening and Cloning

After a HAT selection for 2 wk, screening for antibody production was performed with a solid phase method (28), using biotinylated antimouse IgG antibody (Vector Laboratories, Inc., Burlingame, CA), avidin D (Vector), biotinylated horse radish peroxidase (Vector), and tyramin (Tokyo Kasei Industries Ltd., Tokyo, Jpn). Positive wells were cloned three times by a limiting dilution method (21). Final cloning yielded two hybridoma clones capable of producing antibody against HLF, designated as KDS 101-1 and KDS 101-2, or one clone for BLF, designated as KDS 301-1.

Recovery of Antibody

The antibody was recovered and purified according to StaeheIin et al. (26). Approximately 1 × 10^8 viable hybridoma cells suspended in 5 ml of PBS were injected into the abdominal cavity of 10 BALB/c mice. The ascitic fluid recovered on the d 7 to 10 after the injection was centrifuged to remove cells and debris, and then the supernatant was clarified at 25,000 × g for 90 min at 4°C. The antibody was recovered from the supernatant by precipitation with saturated ammonium sulfate and ion-exchange chromatography on DEAE-cellulose (DE-52; Whatman Chemical Separation Ltd., Maidstone, Kent, UK) with a linear gradient of NaCl; eluents used were 0.02 M Tris-HCl buffers (pH 7.9) containing 0.04 and 0.5 M of NaCl. The McAb was precipitated with an equal volume of saturated ammonium sulfate, dissolved in 0.2 M sodium bicarbonate containing 0.3 M NaCl, dialyzed against the same buffer, and then stored at −20°C until used. One milliliter of ascitic fluid contained approximately 5 to 6 mg of McAb.

Preparation of Affinity Column

An affinity gel coupled with McAb was prepared according to StaeheIin et al. (26). Briefly, Affigel-10 (BioRad Laboratories, Inc., Richmond, CA) activated with isopropanol was mixed with an equal volume of purified McAb solution (20 to 25 mg/ml), stirring gently at
4°C overnight. After excess McAb was removed by centrifugation, unreacted sites of the gel were blocked with .1 M ethanolamine-HCl (pH 8). Approximately 39 mg of HLF-McAb was incorporated into 5 ml of gel (column H), while column B was prepared by packing 1 ml of gel bound by 20 mg of BLF-McAb.

Isolation of Lactoferrin

The affinity gel equilibrated with PBS was packed into a column (13 mm × 70 mm). After each sample was applied on top of the gel, components that did not interact with the McAb were subsequently washed out with PBS, .01 M phosphate buffer (pH 7.2) containing .5 M NaCl, and .15 M NaCl. Thereafter, LF was eluted with a solution of acetate (.2 M) and NaCl (.15 M) at pH 2.7. The pH of eluate was immediately adjusted to 7 with NaOH. The LF fraction was dialyzed against deionized water and then freeze-dried.

Double Immunodiffusion

Double immunodiffusion was performed according to the method described by Ouchterlony (22). Antiserum to mouse γ-globulin was purchased from Cappel Laboratories, Inc. (West Chester, PA); antiserum to HLF was prepared by immunization of BALB/c mice with HLF purified using our affinity column at weekly intervals for 3 wk.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to Laemmli (18), using 11% acrylamide in the presence of .1% SDS. Proteins were stained with Coomassie brilliant blue and purity determined with a densitometer ACD-18 (Gelman Sciences, Inc., Ann Arbor, MI).

Determination of Protein Concentration

Protein concentration was determined spectrophotometrically at 595 nm with a protein assay dye reagent (BioRad) with highly purified BLF as a standard.

Iron Content

Iron content was determined by the bathophenanthroline method (2, 14).

Iron-Binding Capacity

The iron-binding capacity was determined by the modified method of Woodworth (30). Lactoferrin was mixed with monoferrinotriacetate complex in the presence of sodium bicarbonate; optical density at 465 nm was measured. Because $E_{1%}^{1%} cm$ of iron saturated LF at 465 nm is .58 (19), the iron-binding capacity was determined by the ratio of measured $E_{1%}^{1%} cm$ to .58.

RESULTS

Recovery of Lactoferrin

Given amounts of LF standards (affinity purified) were passed through the affinity column to determine yield of recovery. Table 1 summarizes the recoveries observed for HLF and BLF through columns H and B, respectively. Crossreactions between BLF and HLF-McAb or HLF and BLF-McAb did not occur (data not shown). Recoveries of HLF and BLF were 98 and 97%, respectively, indicating that both columns were capable of recovering highly pure LF through a single passage.

Recovery of Human Lactoferrin from Human Colostrum

Figure 1 shows the elution pattern of defatted human colostrum through column H. The double immunodiffusion pattern with anti-HLF serum is shown in Figure 2. Peak 3 clearly showed a spur with anti-HLF serum, indicating that HLF was eluted in this peak. The peak 1 seemed to represent components unreacted with the McAb, since no spur was detected.

Purity of Recovered Human Lactoferrin

The SDS-PAGE pattern of recovered HLF is shown in Figure 3. The pattern was basically the same as that of commercial HLF; purity was densitometrically 98%.

Efficiency of Immobilized Bovine Lactoferrin

The number of available binding sites to BLF of immobilized BLF-McAb was determined to evaluate the maximum amount of BLF recoverable through column B. Required amounts of BLF (97% purity) were passed through the column B; eluted BLF was de-
TABLE 1. Lactoferrin (LF) recovery.

<table>
<thead>
<tr>
<th>Immobilized McAb</th>
<th>HLF-McAb Column H</th>
<th>BLF-McAb Column B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of immobilized McAb, mg</td>
<td>39</td>
<td>20</td>
</tr>
<tr>
<td>Amount of LF applied, mg</td>
<td>(purity 98%)</td>
<td>(purity 97%)</td>
</tr>
<tr>
<td>Amount of LF recovered, mg</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Yield, %</td>
<td>(19.2/20 X .98)</td>
<td>(9.4/10 X .97)</td>
</tr>
</tbody>
</table>

\[1\] HLF = Human lactoferrin, BLF = bovine lactoferrin, McAb = monoclonal antibodies.

determined by the protein assay. As shown in Figure 4, up to 7.6 mg of all BLF applied onto the column were bound to 20 mg of the immobilized antibodies; the amount of unreacted BLF was linearly increased beyond 7.6 mg of applied BLF. Because IgG possesses two binding sites and molecular weights of IgG and LF are 150K (29) and 78K (15), respectively, 1 mg of IgG can bind 1.04 mg of LF. Thus, an apparent efficiency of immobilized BLF-McAb was 36.5% (7.6 x 100/20.8).

Isolation of Bovine Lactoferrin

Elution patterns of bovine milks through column B were similar to that of human colostrum (Figure 1). Table 2 summarizes recoveries of BLF from various milk products. These results agree with those of Korhonen et al. (16), reporting LF contents of matured milk ranging from .03 to .49 mg/ml. Although raw skim milk yielded .12 mg/ml of BLF, pasteurized skim milk contained only half or quarter as much. The recovery from skim milk decreased with an increase in pasteurization temperature, perhaps due to an alteration of the structure of antigenic determinants caused by partial heat denaturation of BLF. However, there was no significant change in the recovery of BLF from cheese whey upon pasteurization at different temperatures.
proteins since LF is highly reactive as suggested by Hekman (11).

Iron Content and Iron-Binding Capacity

Iron content and iron-binding capacity of HLF and BLF recovered through the affinity columns are indicated in Table 3. The iron content of HLF from human colostrum was .14 mg/g protein; that of BLF ranged from .17 to .21 mg/g depending on the origin of the protein. Iron-binding capacity varied considerably with the origin of the lactoferrins. The highest capacity was seen in LF obtained from raw skim milk (99%), whereas BLF from skim milk pasteurized at 70°C for 30 min showed the lowest (43%). The iron-binding capacity decreased with increasing pasteurization temperature, suggesting partial denaturation of LF.

DISCUSSION

An affinity column of immobilized LF-McAb achieved a reasonable recovery of highly pure LF by a single passage of defatted milk. Advantages of this method are the high purity and reasonable recovery of the product and the simplicity of the technique compared with conventional ion-exchange method. Purity can reach 97% (Table 2); thus, the affinity method

Purity of Recovered Bovine Lactoferrin

The SDS-PAGE patterns of recovered BLF are shown in Figure 5. The purity of each specimen is indicated in Table 2. Although the major component was BLF, some impurities were detected, depending on preparation and milk sources. A primary concern was whether mouse γ-globulin contaminates the recovered BLF, because contamination is unfavorable in use of recovered BLF as a food ingredient or pharmaceutical. As shown in Figure 6, double immunodiffusion between the recovered BLF and antimouse γ-globulin serum formed no spur, thus suggesting no contamination of mouse γ-globulin in the recovered BLF. Bovine lactoferrin recovered from raw skim milk demonstrated highest purity (97%); however, the purity appeared to decrease with increased pasteurization temperature. Therefore, the contamination might be attributed to heat-induced interaction between LF and milk

Figure 3. The SDS-PAGE pattern of commercial human lactoferrin (HLF) (A) and recovered HLF (B).

Figure 4. Relationship between the amount of bovine lactoferrin (BLF) applied onto column B and that of BLF unbound to immobilized BLF-monoclonal antibodies (McAb). Twenty milligrams of BLF-McAb was immobilized.
ONE-STEP ISOLATION OF LACTOFERRIN

Figure 5. The SDS-PAGE pattern of raw skim milk (A), recovered bovine lactoferrin (BLF) from raw skim milk (B), from skim milk pasteurized at 65°C (C), from skim milk pasteurized at 70°C (D), from cheese whey (E), and from cheese whey pasteurized at 65°C (F), and cheese whey (G). α-La = α-Lactalbumin; β-Lg = β-lactoglobulin.

The one-step isolation of lactoferrin described is comparable to the one-step chromatography procedure (on heparin-Sepharose) reported by Bläckberg and Hernell (4). Although repeated chromatography increases the purity of LF, the complicated process reduces the yield and jeopardizes reutilization of other valuable components in milk as food materials because of the addition of salts. Disadvantages of the immunoaffinity column are costs of McAb ($1000/g McAb) and the affinity gel. However, production cost of McAb will be reduced with the development of in vitro cell culture technology. An important aspect in the reduction of the production costs of LF is maintenance of the immobilized antibody activity. Column B (stored with .02% of sodium azide at 4°C) has been used over 300 times without any loss in activity. Thus, use of this technique on a large scale represents a future technology for the isolation of BLF on an industrial scale.

Raw skim milk appears to be the most favorable source of LF for purity, iron-binding capacity, and availability. As far as the preservation of the affinity column is concerned,

Figure 6. Double immunodiffusion pattern of recovered bovine lactoferrin (BLF) from raw skim milk (A), from pasteurized skim milk (B), from cheese whey (C), from pasteurized cheese whey (D), and BLF-monodonal antibodies (McAb) (E) against antimouse γ-globulin serum (N).
TABLE 2. Recovery of bovine lactoferrin.

<table>
<thead>
<tr>
<th>Applied sample</th>
<th>Pasteurized skim milk</th>
<th>Pasteurized cheese whey, 65°C</th>
<th>Pasteurized cheese whey, 70°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw skim milk</td>
<td>65°C</td>
<td>70°C</td>
</tr>
<tr>
<td>Sample volume (S), ml</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Protein recovered (P), mg</td>
<td>3</td>
<td>1.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Purity, %</td>
<td>97</td>
<td>88</td>
<td>74</td>
</tr>
</tbody>
</table>

1 Pasteurized for 30 min.
2 Pasteurized for 30 min.
3 Pasteurized for 20 s.
4 Pasteurized for 30 min.

TABLE 3. Iron content and iron-binding capacity of lactoferrin (LF).

<table>
<thead>
<tr>
<th>Source</th>
<th>HLF1 Colostrum</th>
<th>Raw skim milk</th>
<th>Pasteurized skim milk</th>
<th>Pasteurized cheese whey</th>
<th>Pasteurized cheese whey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron content, mg/g protein</td>
<td>.14</td>
<td>.17</td>
<td>.18</td>
<td>.20</td>
<td>.21</td>
</tr>
<tr>
<td>Maximum amount of bound, mg/g protein</td>
<td>1.36</td>
<td>1.42</td>
<td>1.22</td>
<td>1.30</td>
<td>1.18</td>
</tr>
<tr>
<td>Iron binding capacity, %</td>
<td>93</td>
<td>99</td>
<td>84</td>
<td>91</td>
<td>82</td>
</tr>
</tbody>
</table>

1 Total amount of saturated iron of human lactoferrin (HLF) is 1.47 mg/g protein.
2 Total amount of saturated iron of bovine lactoferrin (BLF) is 1.44 mg/g protein.
3 Pasteurized at 65°C for 30 min.
4 Pasteurized at 70°C for 30 min.

Pasteurized skim milk and cheese whey might be preferable because fewer microorganisms are present. Thus, pasteurized skim milk and cheese whey might be alternative LF sources; however, because purity, recovery, and iron-binding capacity decrease with increased pasteurization temperature (Tables 2, 3), it is desirable to pasteurize under milk conditions.

Iron-binding capacity of LF is of critical importance in its biological functions. The difference between the maximum amount of iron bound to LF and the iron content of recovered LF would affect the bacteriostatic effect, since LF develops the effect by taking iron away from microorganisms (7). Growth-promoting effect of LF on lymphocytes cultured in serum-free chemically defined media is attributed to the transportation of free iron in the medium to the cell surface via LF (10). A bactericidal function of neutrophils is enhanced in the presence of iron-saturated LF due to an increase in the production of hydroxyl radicals 5000-fold (3). Because the iron-binding capacity of LF recovered from raw skim milk is nearly perfect (Table 3), development of large scale immunoaffinity columns will be highly conducive to the use of LF in foods, chemicals, and pharmaceuticals.
ACKNOWLEDGMENT

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