ABSTRACT

Heat-induced enthalpy changes in different forms of bovine lactoferrin in water were examined by differential scanning calorimetry. Two thermal transitions with varying enthalpies were observed, depending on the iron-binding status of the protein. Iron-saturated lactoferrin was more resistant to heat-induced changes than was the apolactoferrin. Native lactoferrin had two transitional peaks, and pasteurization affected only the low temperature transition. Iron-saturated lactoferrin revealed a single transitional peak that was resistant to pasteurization. However, both protein forms were completely denatured by UHT. The effect of pasteurization and UHT on the protein interaction capacity with bacteria was examined in a $^{125}$I-labeled lactoferrin binding-inhibition assay. The ability of native and iron-saturated lactoferrins to bind various bacterial species was unaffected by pasteurization. However, UHT treatment decreased this interaction capacity. Native lactoferrins, both unheated and pasteurized, showed similar antibacterial properties and moderately inhibited *Escherichia coli*. However, this inhibitory capacity was lost after UHT treatment. Finally, iron-saturated lactoferrin did not inhibit bacterial growth; neither pasteurization nor UHT could change this property. Thus, UHT seems to affect structural as well as certain biological properties of both native and iron-saturated bovine lactoferrins, and pasteurization seems to be a treatment of choice for products containing this protein.

(Key words: antibacterial activity, lactoferrin, heat treatment, calorimetry)

INTRODUCTION

Milk inhibits the growth of various intestinal pathogenic bacteria and protects children against gastroenteritis (7). Lactoferrin (Lf) is one of the potent antimicrobial agents in milk (23). This iron-binding protein is also present at a high concentration in exocrine secretions that bathe the mucosal surface and in the specific granules of polymorphonuclear leukocytes (18). A role for Lf has been suggested in...
the regulation of intestinal iron absorption (9) and tissue protection during inflammatory reactions (27). Furthermore, the involvement of Lf has been proposed in various physiological pathways, such as myelopoesis, antigen processing, and antibody production (6). During many such processes, Lf binds to specific receptors on pertinent eucaryotic cells.

Lactoferrin could elicit an enhanced antimicrobial effect by adsorbing to the bacterial surface (3, 10, 11). Such interaction seems to be specific to *Escherichia coli* and other members of the Enterobacteriaceae family (13, 14, 20, 26). The Lf-binding components of these bacteria were recently identified as a special class of pore-forming outer membrane proteins. The carbohydrate moiety in lipopolysaccharide may shield porins and protect certain bacteria from Lf interaction (12). The accumulating evidence on the role of Lf in preimmune host defense has encouraged various laboratories to explore a possible usage of this milk protein in infant foods and pharmaceutical products.

Bovine milk is a common and frequently available reservoir for Lf. Bovine Lf (bLf) occurs in the range of .1 of .3 mg/ml in normal milk and 2 to 5 mg/ml in colostrum (29). This 83-kDa glycoprotein is composed of a single polypeptide chain with 17 disulfide bridges (22). The three-dimensional structural arrangement divides bLf in two lobes of equal size, each with a capacity to bind reversibly one Fe³⁺ ion with high affinity in cooperation with a HCO₃⁻ ion (2, 17).

Ruegg et al. (24) studied the thermal behavior of bLf in simulated milk ultrafiltrate at pH 6.7, by differential scanning calorimetry (DSC), and reported a single peak of denaturation for iron-free, bovine apolactoferrin (apo-bLf) and two peaks of denaturation for iron-saturated bLf (Fe-bLf). Abe et al. (1) suggested that bLf was thermostable under acidic conditions, at pH 4.0 in particular. Saito et al. (25) reported an increased antimicrobial activity of bLf after heat treatment at 120°C for 15 min, at pH 2.0, and claimed that an active peptide from bLf was responsible for this effect.

In this study, we have aimed to measure by DSC the enthalpy changes in different forms of bLf heated at various temperatures in water. Furthermore, the capacity of UHT-treated and pasteurized bLf to bind to bacterial cells and to inhibit bacterial growth was investigated.

MATERIALS AND METHODS

bLf Preparations

The bLf was isolated from milk serum by an industrial-scale process (8). Purity of the protein was determined by ion-exchange (Mono-Q column; Pharmacia AB, Uppsala, Sweden) and molecular sieve (TSKG4000SW; LKB Produkter AB, Bromma, Sweden) HPLC.

The elemental analysis for iron was performed using an atomic absorption spectrophotometer (Model 560; Perkin-Elmer Corp., Norwalk, CT). The unmodified bLf isolated from whey (8) contained 252 μg of iron/g of dry weight and was named the native form. To obtain an apo-bLf form, bLf was mixed with .1 M citric acid until pH 2.4 was obtained and kept at 5°C for 24 h. The free iron was removed by gel filtration using a Sephadex G-25 column (Pharmacia), and elution was performed with Milli-Q® water (Millipore AB, Vastra Frolunda, Sweden). Further, a crossflow diafiltration against Milli-Q® water was followed using a UF filter (cutoff: 10-kDa; Millipore AB). After pH readjustment to 5.4, the solution was filter-sterilized (cutoff: .22 μm; Millipore AB) and freeze-dried. The degree of iron saturation of apo-bLf was estimated to be 3.5% from determination of iron of 43 μg/g of dry weight. Finally, for the preparation of Fe-bLf, a solution of 10 mM NaHCO₃ was mixed with .1 g/ml of bLf, and the pH was adjusted to 7.8 with NaOH. To this solution, FeCl₃ was added under continuous stirring to a final concentration of 1 mM, and the mixture was kept at 5°C overnight. Free iron was removed by filtration steps as described, and, after readjustment of pH to 7.2, the protein solution was filter-sterilized. This Fe-bLf preparation contained 1058 μg of iron/g of dry weight (87% saturation).

DSC

Thermal denaturation of various bLf forms was studied by DSC (Perkin-Elmer DSC-2) equipped with hermetically sealed, aluminum-coated sample pans (DuPont, Boston, MA). The protein samples were studied in a temperature region of 25 to 100°C at a scanning rate of 10°C/min. The bLf preparations were dissolved in distilled water to a concentration of 36 or 3.6 mg/ml, and the pH was adjusted to
enthalpy was considered as reference. The volume of solvents in the sample and reference pans was 20 μl. The DSC was calibrated with water, indium, and gallium. An assumed baseline was fitted to a polynomial expression, and the transition enthalpy ($\Delta H_{cal}$) was calculated by stepwise integration of the area of the peak. The temperature at the midpoint of unfolding transition was also determined. Values represent means of three to four independent determinations.

**Heat Treatment of bLf and Fe-bLf**

Protein samples were subjected to two types of heat treatment before DSC analyses. In the first treatment, samples were heated in the DSC to 60, 70, 80, 90, or 100°C at a heating rate of 10°C/min and held for 15 s. After a quick cooling to 25°C in the DSC, the samples were rescanned to 100°C by DSC.

In the second type of treatment, samples were heated in a continuous flow system to 72 or 135°C. Solutions (36 mg/ml) of bLf and Fe-bLf were pasteurized at 72°C for 15 s. The protein solution was diluted to 3.6 mg/ml for UHT treatment (135°C for 4 s) in order to prevent protein gelation in the tube during heating. Heat treatments were in a stainless steel tube (length, 100 mm; diameter, 2 mm; thickness, .5 mm), immersed in a temperature-controlled oil bath, and extended through an ice water bath. Samples were continuously pumped (type FR1; Lewa, Herbert Ott GmbH, Leonberg, Germany) at 10 bar through this tube, and heating time was controlled by flow rate of the solution. The temperature was measured during heating and cooling processes by using thermoelements. The enthalpies of different heat-treated samples were compared with unheated samples, and a reduction in the enthalpy was considered as an indication of protein denaturation.

**125I-Labeled bLf Binding-Inhibition Assay**

The capacity of pasteurized and UHT-treated bLf and Fe-bLf to interact with bacterial cells was examined in a $\text{125I-labeled bLf}$ binding-inhibition assay using previously characterized strains of bacteria (13, 14, 19, 20, 26). The bLf was labeled with Na$^{125}$I (specific activity 629 GBq/mg) (DuPont Scandinavia AB, Stockholm, Sweden) using Iodobeads (Pierce Chemicals Co., Rockford, IL) (16). Assay was performed as described earlier by Naidu et al. (19, 20); in brief, increasing amounts (.1 to 1.2 mg/ml) of unlabeled bLf or Fe-bLf diluted in .1 ml of .15 M phosphate-buffered saline were mixed with an equal volume of the same buffer containing about 8 ng of $\text{125I-labeled bLf}$ (specific activity, .16 MBq/μg). In the binding assay, approximately $10^9$ bacteria (cell density was optically adjusted at 600 nm) grown in special peptone-yeast extract (SPYE) broth, pH 7.3 (Malthus Instruments Ltd., West Sussex, England), were added (final volume, .3 ml) and thoroughly mixed. After 1 h of incubation at 37°C, the binding reaction was stopped by addition of 2 ml of ice-cold phosphate-buffered saline, pH 7.2 (containing .1% Tween-20). The suspension was centrifuged at 5300 × g for 15 min, and the supernate was aspirated. Radioactivity bound to the bacterial pellet was measured in a gamma counter (Clinigamma 1272; LKB Wallac, Turku, Finland). The binding of $\text{125I-labeled bLf}$ to bacteria in phosphate-buffered saline in the absence of unlabeled bLf was considered to be 100% (control), and, in the presence of unlabeled bLf, the binding was expressed in percentage relative to control.

**Antibacterial Assay**

The effect of bLf on bacterial growth was estimated by measuring changes in conductance of the cultivation media using a Malthus AT system® equipped with a built-in computer system (Malthus Instruments Ltd., West Sussex, England). The assay was performed in special glass tubes (10-ml volume) containing SPYE broth with or without bLf (1.6 to 5 mg/ml). The tubes were inoculated with E. coli H10407[LF] (1.4 × 10⁴ bacteria/ml) from an overnight SPYE broth culture (final volume: 3 ml) and sealed with special screw caps fitted with platinum electrodes and incubated at 37°C. The substrate utilization and production of metabolites by the bacteria was continuously measured as the rate of change in conductance in microsiemens per hour of the culture media. The change in conductance was referred to as metabolic activity. The following definitions were used for the data interpretation: detection time (hours) was the time required to initiate a detectable change in conductance (i.e., the sensitivity of the assay); the inability of bacteria to cause a change in con-
ductance was considered as metabolic inhibition; bacteriostasis was the difference in detection times variance between bacterial growth in the absence and presence of bLf; and metabolic activity (microsiemens per hour) was the rate of change in the conductance measured from the slopes of the growth curves.

RESULTS

Thermal Behavior of Different Forms of bLf

Thermal denaturation of bLf, apo-bLf, and Fe-bLf (dissolved in water, pH 7.2) was examined by DSC (Figure 1). The DSC thermograms of bLf showed two transitions of varying enthalpies, depending on the iron status of the protein (Table 1). Thermogram of the native bLf showed two denaturation temperatures (i.e., peak 1 at 65°C and peak 2 at 92°C). However, apo-bLf and Fe-bLf showed prominent thermal denaturation peaks at 71°C (corresponding to peak 1 of bLf) and 93°C (corresponding to peak 2 of bLf), respectively. None of the three forms of bLf showed any peaks after rescanning.

Thermal Behavior of Preheated bLf and Fe-bLf

The effect of preheating at various temperatures on bLf and Fe-bLf was examined (Figure 2; Table 2). The DSC thermogram profile of bLf did not change after heating at 60°C for 15 s. However, heat treatment of bLf at 70 and 80°C for 15 s has reduced the ΔHcal of the peak 1 to 3.7 and 1.9 J/g, respectively, compared with the unheated bLf (14.2 J/g). Heat treatment slightly affected the enthalpy of peak 2. Thus, preheating of bLf to 70 and 80°C caused a protein denaturation of 50 and 60%, respectively. Finally after heating to 90°C for 15 s, bLf showed no transitional peaks and was completely denatured. However, heat treatment at 60°C slightly affected the thermal transition of Fe-bLf, and 70 and 80°C treatments caused a 22 to 24% denaturation. Heat treatment at 90°C denatured 85% of the Fe-bLf, and, at 100°C, the proteins were completely uncoiled.

Thermal Behavior of Pasteurized and UHT-Treated bLf and Fe-bLf

Solutions (36 mg/ml) of bLf and Fe-bLf were pasteurized at 72°C for 15 s and then examined by DSC. In the pasteurized bLf, the ΔHcal of peak 1 in the thermogram was reduced by 7 to 9 J/g (40 to 50% denaturation), and the ΔHcal of peak 2 was slightly affected. In contrast, pasteurization did not affect the ΔHcal of Fe-bLf. Solutions of bLf and Fe-bLf diluted to 3.6 mg/ml received UHT treatment (135°C for 4 s) and were examined by DSC. Both samples were completely denatured after UHT treatment, as the proteins aggregated and the solutions became opaque.

Interaction of Heat-Treated bLf and Fe-bLf with Bacteria

The ability of heat-treated forms of bLf to interact with bacteria was examined in a

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125I-labeled bLf binding-inhibition assay, using *E. coli* strain H10407[LF] (Figure 3). Unheated and pasteurized bLf demonstrated comparable concentration-dependent, binding-inhibition curves. The UHT-treated bLf was less effective and required a twofold higher concentration (ca. 0.4 mg/ml) to elicit a 50% binding-inhibition compared to the unheated protein. The interaction of Fe-saturated bLf with bacteria was similar to native bLf and did not change after pasteurization. However, the UHT treatment markedly decreased the blocking capacity of Fe-bLf, and a fourfold higher concentration (ca. 0.8 mg/ml) was required to cause a 50% effect compared with unheated protein.

Furthermore, the ability of heat-treated bLf preparations to interact with bacteria was examined with one Gram-positive and four different species of Gram-negative bacteria (Table 3). Pasteurization did not change the blocking capacity of bLf and Fe-bLf in all five bacterial species tested. However, inhibition capacity of the UHT-treated bLf decreased (above twofold) in *Salmonella typhimurium* and *Staphylococcus aureus*, but slightly affected in *Shigella flexneri*, *Aeromonas hydrophila*, and *Yersinia enterocolitica*. However, the UHT-treated Fe-bLf had a reduced capacity to block the 125I-bLf binding to *Staph. aureus* (eightfold), *Shig. flexneri* (threefold), and *Sal. typhimurium* (twofold); however, the protein could block the binding in *A. hydrophila* and *Y. enterocolitica* comparable with the unheated Fe-bLf.

**Antibacterial Activity of Heat-Treated bLf and Fe-bLf**

The effect of various heat-treated forms of bLf on the growth of *E. coli* H10407[LF] was examined (Figure 4; Table 4). In SPYE media, strain H10407[LF] grew at a metabolic rate of 300 ± 14 µS/h with a detection time of 3.5 ± 0.4 h (four experiments), and the cells could bind about 22% of the 125I-labeled bLf. The incorporation of unheated or pasteurized bLf (4 mg/ml) in SPYE media inhibited the metabolic rate by about 80% and prolonged the detection time, causing a bacteriostasis of about 3 h. The UHT-treated bLf slightly reduced the bacterial metabolic rate (31%); however, this protein did not cause any bacteriostasis. Addition of Fe-bLf—untreated, pasteurized, or UHT—did not affect the growth of strain H10407[LF] in SPYE media.

**DISCUSSION**

The present DSC experiments indicated two transitional peaks of thermal denaturation for bLf. The transitions at low and high tempera-
TABLE 1. Denaturation temperature (T\text{\textsc{m}}\text{\textsc{x}}) and enthalpy (\Delta H\text{\textsc{c}}\text{\textsc{a}}\text{\textsc{l}}) of different forms of bovine lactoferrin (bLf) in water at pH 7.2.

<table>
<thead>
<tr>
<th>bLf Form\textsuperscript{1}</th>
<th>T\text{\textsc{m}}\text{\textsc{x}}\textsuperscript{1} (°C)</th>
<th>T\text{\textsc{m}}\text{\textsc{x}}\textsuperscript{2} (°C)</th>
<th>\Delta H\text{\textsc{c}}\text{\textsc{a}}\text{\textsc{l}}\textsuperscript{1} (J/g)</th>
<th>\Delta H\text{\textsc{c}}\text{\textsc{a}}\text{\textsc{l}}\textsuperscript{2} (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>apo-bLf</td>
<td>(\bar{x}) = 71 .3</td>
<td>(\bar{x}) = 90 .3</td>
<td>(\bar{x}) = 12 .4</td>
<td>(\bar{x}) = 2 .2</td>
</tr>
<tr>
<td>bLf</td>
<td>(\bar{x}) = 65 .5</td>
<td>(\bar{x}) = 92 .5</td>
<td>(\bar{x}) = 14 .4</td>
<td>(\bar{x}) = 11 .5</td>
</tr>
<tr>
<td>Fe-bLf\textsuperscript{2}</td>
<td>(\bar{x}) = 65 .3</td>
<td>(\bar{x}) = 93 .3</td>
<td>(\bar{x}) = 2 .2</td>
<td>(\bar{x}) = 37 .1</td>
</tr>
</tbody>
</table>

\textsuperscript{1}apo-bLf = Bovine apolactoferrin (iron-free), Fe-bLf = iron-saturated bLf.

\textsuperscript{2}Four replicates.

Table entries corresponded to apo-bLf and Fe-bLf, respectively. In an earlier study, Paulsson and Visser (21) suggested that the heat-induced unfolding of the two lobes of native bLf are independent. The enthalpy of the transition increased at high temperature (92°C) when the iron content in the preparation increased. This transition indicates the unfolding of the iron-containing parts of bLf. The data taken together indicate that the binding of iron stabilized the protein structure against heat treatment. A slightly higher ionic strength of apo-bLf solution might have caused a small elevation in the denaturation temperature of peak 1 compared with the corresponding peak of native bLf. The thermal denaturation (protein unfolding to a random coil structure) of bLf, apo-bLf, and Fe-bLf seemed to be irreversible be-

Figure 3. Blockage of \(^{125}\text{I}\)-labeled bovine lactoferrin (bLf) interaction with Escherichia coli H10407 Lf by unlabeled bLf and iron-saturated bLf (Fe-bLf). Increasing amounts of pasteurized (○) or UHT-treated (△) proteins were mixed with labeled ligand and incubated with bacterial cells. Untreated protein sample served as control (●). The bacterial interaction with labeled protein in phosphate-buffered saline (in the absence of unlabeled protein) was considered to be 100%.

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TABLE 2. Degree of denaturation\(^1\) of bovine lactoferrin (bLf) and iron-saturated bLf (Fe-bLf) after preheat treatment at various temperatures.\(^2\)

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Peak 1 (\bar{X} \pm SD)</th>
<th>Peak 2 (\bar{X} \pm SD)</th>
<th>Total (\bar{X} \pm SD)</th>
<th>Total denaturation of Fe-bLf (\bar{X} \pm SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>10 2</td>
<td>10 1</td>
<td>10 1</td>
<td>10 2</td>
</tr>
<tr>
<td>70</td>
<td>14 3</td>
<td>12 2</td>
<td>16 4</td>
<td>18 3</td>
</tr>
<tr>
<td>80</td>
<td>21 3</td>
<td>18 2</td>
<td>24 3</td>
<td>26 3</td>
</tr>
<tr>
<td>90</td>
<td>100 0</td>
<td>100 0</td>
<td>100 0</td>
<td>100 0</td>
</tr>
<tr>
<td>100</td>
<td>100 0</td>
<td>100 0</td>
<td>100 0</td>
<td>100 0</td>
</tr>
</tbody>
</table>

\(^1\)Percentage denaturation was calculated by the following equation: \([1 - \Delta H_{cal} \text{ of the preheated sample}/\Delta H_{cal} \text{ of the control}] \times 100\), where \(\Delta H_{cal} = \text{denaturation enthalpy}\).

\(^2\)Thermal denaturation of aqueous protein solutions (pH 7.2) was studied by differential scanning calorimetry. Samples not preheated served as controls. Three replicates.

cause the denatured bLf after cooling failed to demonstrate any peaks in the thermogram during DSC rescanning. Ruegg et al. \((24)\) also reported a similar effect for Fe-bLf; however, the apo-bLf showed a reversible denaturation in simulated milk ultrafiltrate \((40 \text{ to } 50\% \text{ renaturation})\) in their study. This observation was also reported by Baer et al. \((5)\) using fluorimetric and immunological analyses. A lower denaturation of pasteurized bLf than the preheated bLf in DSC may possibly be due to the effect of rapid heat treatment in pasteurization process.

We have recently shown that the native bLf dissolved in distilled water at pH 4 to 9 was more thermostable at basic pH and that the thermal denaturation was also dependent on ionic strength of the solution \((21)\). However, at low pH \((<\text{pH 3.0})\), bLf showed no transition to indicate a total unfolding of the protein prior to

TABLE 3. Effect of temperature of heat treatment of native and Fe-saturated bovine lactoferrins (Fe-bLf) on their interaction capacity with different bacteria.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>bLf Inhibition of (^{125}\text{I}-\text{labeled bLf binding}(^2)</th>
<th>Fe-bLf Inhibition of (^{125}\text{I}-\text{labeled bLf binding}(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unheated Pasteurized UHT</td>
<td>Unheated Pasteurized UHT</td>
</tr>
<tr>
<td></td>
<td>(\bar{X} \pm SD) (\bar{X} \pm SD) (\bar{X} \pm SD)</td>
<td>(\bar{X} \pm SD) (\bar{X} \pm SD) (\bar{X} \pm SD)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H10407[LF]</td>
<td>50 3</td>
<td>50 3</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>68 3</td>
<td>68 4</td>
</tr>
<tr>
<td>R10</td>
<td>30 2</td>
<td>26 2</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>47 6</td>
<td>41 3</td>
</tr>
<tr>
<td>SPL1070-15</td>
<td>71 4</td>
<td>67 2</td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>54 2</td>
<td>48 2</td>
</tr>
<tr>
<td>CCUG14551</td>
<td>50 3</td>
<td>50 3</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>68 3</td>
<td>68 3</td>
</tr>
<tr>
<td>Y162</td>
<td>37 3</td>
<td>37 3</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>23 1</td>
<td>23 1</td>
</tr>
<tr>
<td>SA43</td>
<td>48 3</td>
<td>48 3</td>
</tr>
</tbody>
</table>

\(^1\)Differently heat-treated solutions of bLf or Fe-bLf in .1 ml of phosphate-buffered saline containing .1 mg of protein were mixed with ca. 8 ng of \(^{125}\text{I}-\text{labeled bLf and 10}^9\) bacterial cells. Assay was performed as described. Means were expressed as percentages, considering the inhibition of binding in phosphate-buffered saline (control) as zero. Three replicates. bLf = Bovine lactoferrin.
heating (21). Conversely, Abe et al. (1) reported a high thermostability of apo-bLf at pH 4.0. However, this group has monitored only the protein homogeneity by HPLC techniques after heat treatments and did not measure thermal transitions or unfolding of the protein.

The adsorption of bLf to the microbial surface seems to be important to cause bacteriostatic, bactericidal, and opsonic effects (2, 10, 15). Various microbial pathogens demonstrate specific Lf-binding components on their cell surface (12, 14, 19, 20, 26). Our binding-inhibition studies revealed that the interaction capacity of bLf and Fe-bLf with four different species of Gram-negative bacteria and a Gram-positive S. aureus was unaffected by pasteurization and was reduced to different magnitudes by UHT treatment. Thus, the protein denaturation by UHT treatment might have altered the binding domains on the bLf, which would be consistent with earlier observations that the interaction was dependent on the protein conformation because complexes of Lf and bacterial outer membrane proteins were dissociable by chaotropic agents such as potassium thiocyanate (26).

The unheated and pasteurized bLf preparations showed similar antibacterial properties and caused an effective metabolic inhibition with a moderate bacteriostasis. The UHT-treated bLf caused only a slight reduction in the bacterial metabolism and failed to elicit bacteriostasis. Recent studies have suggested that a correlation exists between the degree of Lf binding to bacteria and antimicrobial activity (28). Therefore, loss in the antimicrobial activity of UHT-treated bLf could possibly be due to a reduced interaction capacity of the protein with the bacteria from heat-induced conformational changes. However, Fe-bLf (untreated, pasteurized, or UHT treated) did not affect the bacterial growth, which might be due to the inability of these proteins to chelate iron (17). Finally, the protein conformation of the apo-bLf is important for the antibacterial effect (2, 4).

<table>
<thead>
<tr>
<th>Sample and treatment</th>
<th>Bacteriostasis² (%)</th>
<th>Metabolic activity³ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>bLf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unheated</td>
<td>2.6</td>
<td>13</td>
</tr>
<tr>
<td>Pasteurized</td>
<td>3.4</td>
<td>21</td>
</tr>
<tr>
<td>UHT Treated⁴</td>
<td>0.2</td>
<td>66</td>
</tr>
<tr>
<td>Fe-bLf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unheated</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Pasteurized</td>
<td>0</td>
<td>86</td>
</tr>
<tr>
<td>UHT Treated⁴</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

¹Metabolism was measured as change in conductance in microsiemens of the bacterial growth media either in the absence (control) or presence of bovine lactoferrin (bLf) (4 mg/ml). Four replicates.

²Difference in detection times between growth in media with and without bLf.

³Expressed in percentage relative to growth in media with Fe-bLf.

⁴Protein concentration was 1.6 mg/ml in the samples.

Figure 4. Growth of Escherichia coli H10407[bLf] in special peptone-yeast extract broth containing untreated (labeled 1), pasteurized (labeled 2), and UHT-treated (labeled 3) bovine lactoferrin (bLf) or iron-saturated bLf (Fe-bLf). Bacterial growth in media without lactoferrin served as control (labeled C). The bacterial metabolism at 37°C was measured for 24 h, as the change in conductance of growth media, using a Malthus-AT® system (Malthus Instruments Ltd., West Sussex, England).
CONCLUSIONS

In conclusion, we confirmed that the thermal denaturation of bLf in water was dependent on the iron status of the protein. The UHT treatment denatured the protein structure and also diminished the antibacterial properties of bLf. Pasteurization seems to be the method of choice because it did not alter either the bacterial interaction capacity or the antibacterial activity of bLf. However, because Lf has been associated with several biological functions, the effect of heat treatment on these properties remains to be elucidated.

ACKNOWLEDGMENTS

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