Effect of Iron Saturation on the Recovery of Lactoferrin in Rennet Whey Coming from Heat-Treated Skim Milk

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ABSTRACT

This study aimed to determine the effect of thermal treatments on the recovery of lactoferrin in whey coming from rennet-coagulated skim milk. The impact of lactoferrin iron saturation was also assessed using skim milk spiked with different lactoferrin iron forms. The recovery of lactoferrin in the rennet whey fraction was determined by reverse-phase HPLC. One- and 2-dimensional sodium dodecyl sulfate PAGE analyses were performed on rennet curds to characterize the protein interactions involving lactoferrin in heated milk. The extent of lactoferrin recovered in the whey fraction was found to reduce as the heating temperature increased. The binding of iron by lactoferrin improved its thermal stability and its recovery in the whey fraction. Polyacrylamide gel electrophoresis results showed that the association of lactoferrin in the unheated milk rennet curd involved noncovalent interactions, whereas upon heating, lactoferrin also interacted via an intermolecular disulfide link. Depending on the severity of the heat treatment, lactoferrin aggregates with Cys-containing proteins (β -lactoglobulin, α -lactalbumin, α_{s2} -casein, and κ -casein) occurred by intermolecular thiol/disulfide exchange reactions. These noncovalent and covalent interactions explained the lower recovery of lactoferrin in heated milk.

Key words: heat treatment, lactoferrin, iron saturation, whey

INTRODUCTION

Lactoferrin (LF) is an iron-binding glycoprotein in the transferrin family occurring in different external secretions, including milk. The concentration of LF in bovine milk varies with the lactation period. Values between 0.02 and 0.2 g/L have been reported (Steijns

and van Hooijdonk, 2000). Lactoferrin is a single polypeptide chain glycoprotein of about 80 kDa (689 AA) that includes 17 intramolecular disulfide bonds (S–S) but no free Cys residues (Pierce et al., 1991). The protein is folded into 2 analog lobes linked by a short α -helix peptide. Each lobe has 2 domains forming a deep cleft within which a ferric ion (Fe^{3+}) binds reversibly in coordination with a carbonate ion (CO_3^{2-} ; Moore et al., 1997). Lactoferrin is only partially saturated with iron (15 to 20%; Steijns and van Hooijdonk, 2000) in bovine milk. However, depending on the environmental conditions, the protein could adopt an iron-free form (apo-LF) or an iron-loaded form (holo-LF).

Ward et al. (2005) recently discussed the various biological properties ascribed to LF, including antimicrobial, anti-inflammatory, antitumor, immunomodulatory, and bone growth factor activities. The strong potential of LF as a natural bioactive ingredient in food and health products has stressed the development of suitable techniques for its isolation from milk fluids. Lactoferrin has a high isoelectric point (pI), between 8.0 and 9.0 (Shimazaki et al., 1993). The distinct basic character of LF allows its extraction from skim milk or, more commonly, from cheese whey by cation-exchange chromatography. Nowadays, LF isolates of high purity are commercially available. However, the heat treatment of milk before cheese making might denature the protein and reduce its recovery in whey (Smithers et al., 1996).

Resistance of LF to thermal denaturation depends on its iron saturation because the more compact conformation of the holo-LF improves its heat stability compared with the more open apo form (Sanchez et al., 1992; Paulson et al., 1993; Kussendrager, 1994). Sanchez et al. (1992) also suggested that the HTST pasteurization (72°C, 15 s) commonly used in the cheese-making industry has a limited effect on LF denaturation. However, Paulson et al. (1993) reported that HTST pasteurization induces partial denaturation of the native protein, affecting its lower unfolding transition by 40 to 50%, whereas UHT treatment led to a complete denaturation of the protein.

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It was recently observed that the LF thermal aggregation process implies intermolecular thiol/disulfide interchange reactions (Brisson et al., 2007). These reactions are initiated by free thiols likely generated from intramolecular disulfide cleavages, because LF does not possess free Cys residues. The binding of iron was shown to increase LF disulfide bond integrity. The thermal stability of LF has also been studied in more complex systems, in which the denaturation rate of LF was found to increase in the presence of whey proteins (Sanchez et al., 1992; Kussendrager, 1994). Moreover, combined pressure and heat treatments (HTST pasteurization and UHT treatment) also seem to induce LF aggregation in whey protein concentrate solutions (Patel et al., 2004) and raw milk (Nabhan et al., 2004; Patel et al., 2006). The presence of LF in serum protein aggregates coming from heated reconstituted skim milk (90°C, 10 min) has also been reported (Jean et al., 2006). In those cases, the thermal association of LF with other proteins was thought to involve intermolecular disulfide bridges. These interactions might impair LF recovery in cheese whey. However, a recent study suggested that the level of LF recovered in cheese whey depends on the cheese-making procedure rather than on the milk HTST pasteurization treatment (Dupont et al., 2006).

This study aimed to investigate the effect of iron saturation on the thermal association of LF in milk and the impact on its recovery in rennet whey. The experiments were performed on heat-treated skim milk and milk samples spiked with either the apo-, native, or holo-LF forms. One-dimensional (1D) and 2-dimensional (2D) SDS-PAGE analyses were used to characterize the nature of interactions between LF and the other milk proteins present in the rennet curd.

MATERIALS AND METHODS

Isolation of LF

Lactoferrin was isolated from raw skim milk by cation-exchange chromatography based on the method of Uchida et al. (1996). The raw milk (coming from Laiterie Mont St-Hilaire, Parmalat Canada, St-Hyacinthe, Québec, Canada) was skimmed at a temperature of 37°C using a pilot-scale centrifugal separator (Westfalia, type LWA-205, Englewood, NJ), and then refrigerated at a constant temperature of 4°C before being passed through an SP Sepharose Big Beads resin column (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada). The LF fraction was eluted with a 2-step elution consisting of 50 mM phosphate ($\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$) buffers at pH 6.8 containing, respectively, 0.4 M NaCl to elute lactoperoxidase and 0.7 M NaCl to elute LF. The desorbed LF fraction was then extensively dialyzed

Table 1. Physicochemical characteristics of the different lactoferrin iron preparations used in the study

Lactoferrin	Protein (%)	Lactoferrin purity (% of total protein)	Iron saturation (%)
Apo-lactoferrin	94.0	92.4	4.6
Lactoferrin	83.1	93.8	19.2
Holo-lactoferrin	88.4	91.7	100

against Milli-Q purified water (4°C) before being freeze-dried. The isolated native LF is referred to as LF in this article.

Modification of LF Iron Saturation

The previously isolated LF was used as the starting material to prepare LF with modified iron content. Apo-LF was prepared by the method of Mazurier and Spik (1980). The saturation of LF with iron was obtained with a freshly prepared FeNTA solution [9.9 mM $\text{Fe}(\text{NO}_3)_3$ and 8.5 mM nitrilotriacetic acid] according to van Berkel et al. (1995). The excess iron was removed by continuous diafiltration with a 30-kDa molecular weight cutoff spiral-wound ultrafiltration cartridge (Amicon, model S1Y30, Beverly, MA) fitted to a bench-top Amicon ultrafiltration cell (Amicon) and then freeze-dried. The iron saturation status of LF was estimated by the ratio of light absorption at 465 and 280 nm (Hashizume et al., 1987). The protein content was determined by the Dumas method with a Leco FP-528 nitrogen-protein analyzer (Leco, St. Joseph, MI; IDF, 2002) and the purity was assessed by reverse-phase (RP)-HPLC according to the method of Palmano and Elgar (2002) as described below. Characteristics of the different LF iron forms are reported in Table 1.

Milk Supply and Preparation of Concentrated Milk

Raw skim milk was from a local dairy farm. The milk was kept at 4°C before being skimmed at 37°C using a pilot-scale centrifugal separator (Westfalia). Sodium azide was added (0.02%, wt/vol) to the raw skim milk to prevent bacterial growth. The milk was stored at 4°C and used within 2 d.

Sample Preparation and Heat Treatments

In some experiments, raw skim milk was spiked with the different LF iron forms at a protein concentration of 0.25% (wt/vol) and stirred overnight at 4°C. Before thermal treatments, all milk samples (enriched and nonenriched) were allowed to equilibrate at room temperature (2 h). Aliquots of milk samples (5.0 mL) were

transferred into polypropylene centrifuge tubes (17 mm i.d. and 118 mm length; VWR, Mississauga, Ontario, Canada), screw capped, and heated in a water bath at temperatures ranging from 60 to 80°C for 10 min. To decrease the heating time, the samples were placed in an ethylene glycol bath set at 30°C over the target temperature and the milk temperature was monitored with a thermocouple. When the milk approached the targeted temperature (within 3°C), the sample tubes were rapidly transferred into the water bath and held at the desired temperature for a predetermined time (10 min). The times required to reach the target temperature varied between 75 and 135 s in the temperature range tested. Heated samples were then rapidly cooled on ice water and allowed to equilibrate to room temperature (2 h).

Rennet Coagulation of Milk

The heated milk samples were submitted to rennet coagulation to separate the whey fraction from the colloidal phase using the method described by Noh and Richardson (1989) with modifications. Fifty microliters of a 15% (wt/vol) CaCl_2 solution was added to improve coagulation of the heated milk samples briefly before renneting, and the samples were stirred at room temperature for 60 min. The milk was then preheated at 30°C for 20 min, and double-strength rennet (Chymax, Chr. Hansen, Milwaukee, WI) diluted in deionized water (1:10) was added (5 μL). The milk samples were further incubated at 30°C until milk coagulation (~30 min). The coagulated milk samples were cut and then cooked at 40°C for 30 min. Whey was recovered by centrifugation at $3,000 \times g$ for 5 min. Rennet curds were washed twice with deionized water made up to approximately 5 mL and then recentrifuged at $3,000 \times g$ for 5 min.

RP-HPLC

The individual whey protein concentrations in the whey fraction were determined by RP-HPLC according to the method of Palmano and Elgar (2002) using a 1-mL Resource RPC column (Amersham Pharmacia Biotech). Analyses were performed with an HPLC system from Waters (Milford, MA) equipped with 2 pumps (model 600E), a UV-visible detector (model 486) set at 214 nm, and an automatic injector (Hewlett-Packard series 1100, Agilent Technologies, Palo Alto, CA). Data acquisition and analysis were performed using the Millennium 2.1 chromatographic software (Waters). The protein standards were glycomacropeptide, α -LA, LF, BSA, β -LG, and IgG, all from Sigma (St. Louis, MO). Before analysis, aliquots (1 mL) of whey samples were

transferred in microtubes and centrifuged ($16,000 \times g$ for 3 min) before injection to remove the insoluble protein aggregate material. The percentage of LF recovery was determined by the ratio of LF concentration in the whey fraction of the heated sample to the initial concentration ($\% [\text{LF}]_{\text{w}}/[\text{LF}]_{\text{o}}$). Because the HPLC method did not allow us to determine the endogenous LF content in milk directly, the concentration obtained by HPLC analysis for the unheated whey sample was used as the reference. The protein concentration in the unheated whey sample was also used as the reference for the other whey proteins.

PAGE

One-dimensional and 2D SDS-PAGE were performed on the precipitated curd samples. The precipitated rennet curds were dispersed, based on the method of Vasbinder et al. (2003), in 50 mM Tris-HCl buffer, pH 6.8, containing 5% SDS made up to 5 mL. The samples were stirred overnight at room temperature to allow their complete dispersion. The samples were then further diluted in the SDS buffer at ratio of 1:10. β -Mercaptoethanol was added to some samples (1%, vol/vol) and then boiled for 4 min to reduce the disulfide bonds. The protein samples were first analyzed by 1D SDS-PAGE under reducing and nonreducing conditions according to the buffer system of Laemmli (1970). Every sample was diluted 1:2 with a premixed Laemmli sample buffer before electrophoresis (Bio-Rad Laboratories, Hercules, CA). Samples (20 μL) were loaded onto 13.5% gels (1.5 mm thick) for both reducing and nonreducing SDS-PAGE conditions. Electrophoresis of the protein samples was performed with a Mini-Protean III electrophoresis cell system (Bio-Rad Laboratories). The gels were stained with Coomassie Brilliant Blue R-250. The molecular weight markers were myosin (201.1 kDa), β -galactosidase (115.7 kDa), BSA (93.6 kDa), ovalbumin (50.4 kDa), carbonic anhydrase (37.4 kDa), soybean trypsin inhibitor (29.0 kDa), lysozyme (19.4 kDa), and aprotinin (6.9 kDa), all provided by Bio-Rad Laboratories. Two-dimensional SDS-PAGE analyses were performed on some samples according to the technique developed by Havea et al. (1998). The first nonreducing SDS-PAGE dimension was accomplished as described above. A sample gel strip was cut and reduced after the first-dimension electrophoresis and then submitted to a second SDS-PAGE dimension to complete the characterization of interactions occurring in the rennet curd.

RESULTS AND DISCUSSION

Changes in Protein Recovery in Rennet Whey

Figure 1 illustrates the effect of milk heating temperature on the recovery of LF in skim milk rennet whey.

Figure 1. Effect of thermal treatments on the percentage of lactoferrin (LF) recovered ($\% [LF]_w/[LF]_o$) in the rennet whey of skim milk heated at different temperatures (10 min) and analyzed by reverse-phase HPLC. Error bars represent the standard deviation of triplicate independent samples. $[LF]_w$ represents the LF concentration in the heated whey fraction and $[LF]_o$ represents the initial LF concentration in the unheated whey (reference).

Figure 2. Effect of iron saturation on the percentage of lactoferrin (LF) recovered ($\% [LF]_w/[LF]_o$) in the rennet whey fraction from thermally treated (10 min) skim milk enriched (0.25% protein, wt/vol) with holo-LF (black bars), LF (gray bars), and apo-LF (white bars), and analyzed by reverse-phase HPLC. Error bars represent the standard deviation of triplicate independent samples. $[LF]_w$ represents the LF concentration in the heated whey fraction and $[LF]_o$ represents the initial LF concentration in the unheated whey (reference).

In our experiments, the level of LF released in the unheated rennet skim milk whey fraction (181 $\mu\text{g/mL}$) was used to estimate the endogenous LF concentration in the initial skim raw milk. This value was in the range of the LF concentrations reported recently for raw milk (157 to 176 $\mu\text{g/mL}$) as determined by immunoassay techniques (Hagiwara et al., 2003; Chen and Mao, 2004; Dupont et al., 2006). However, our method was able to underestimate the real value in milk because part of the LF could have been retained in the cheese curd, as reported by Dupont et al. (2006). Those authors observed that, depending on the cheese-making procedure, only 19 to 39% of the initial LF was recovered in whey, corresponding to LF concentration values of approximately 25 to 70 $\mu\text{g/mL}$. The large differences compared with our results may be ascribed to the fact that the authors used bacteria starters for acidification of milk prior to rennet coagulation. In fact, it is well known that LF binds to CN and that acid precipitation of milk (pH 4.6) impairs the recovery of LF in whey (Groves, 1960; Nuyens and van Veen, 1999). Moreover, as the heating temperature increased, a progressive decrease in the LF concentration was observed in the coagulated skim milk rennet whey fraction. This result suggests that the thermal treatment induced association of the LF in milk with either the CN micelles or the whey proteins. This probably caused the protein to be retained in the curd or to be expelled in the whey fraction in an aggregated form. No residual LF was

detected in the rennet-treated skim milk whey fraction at 75°C.

To study the influence of LF iron saturation on the recovery of LF in whey, the milk samples were spiked with the different LF iron forms. Changes observed in the level of LF recovered in the thermally treated enriched skim milk whey were also assessed by RP-HPLC (Figure 2). For the nonspiked milks (Figure 1), a sharp decrease in the percentage of LF recovered was observed as the severity of the heat treatment increased. The decline started at around 65°C for the 3 different LF iron forms in enriched milk. However, the recovery of LF in the rennet whey fraction increased with the protein iron content at a temperature above 65°C. As expected, the holo protein was the most heat-stable iron form, whereas the partially saturated LF and the apo protein showed lower stability. These results reinforce the research of Sanchez et al. (1992), who suggested that the increase in holo-LF thermal stability in milk is related to its more compact structure, compared with the more open conformation of the apo protein. The thermal stability of 19% iron-saturated LF was also slightly higher than that of the apo proteins. This was probably due to the more stable holo part of the partially saturated LF, as observed by Kussendrager (1994). These results show that the extent of the LF recovery in whey coming from thermally treated milk depends on its iron saturation.

Figure 3. Effect of LF iron saturation on the percentage of β -LG (A) and α -LA (B) recovered in the rennet whey fraction from thermally treated (10 min) skim milk (dark gray bars) and milk spiked (0.25% protein, wt/vol) with holo-LF (black bars), native LF (gray bars), and apo-LF (white bars), and analyzed by reverse-phase HPLC. Error bars represent the standard deviation of triplicate independent samples.

The RP-HPLC method also allowed us to monitor the influence of heat treatments on the recovery of the 2 major whey proteins (β -LG and α -LA) in the whey fraction. The results are summarized in Figure 3. In skim milk, β -LG was more susceptible to thermal treatments

because a lower level of β -LG was recovered in the whey fraction compared with α -LA. These results reinforce literature reports (Vasbinder et al., 2003). However, a more important decrease in the concentrations of β -LG and α -LA in the whey fraction was observed for more severe heat treatments in milk samples spiked with the different LF iron forms. At 75°C, a clear difference in the percentage of β -LG recovered in the whey of LF-enriched milk was observed, compared with the corresponding skim milks. This result suggests that LF could associate with β -LG in enriched milk. In fact, upon heating the milk, β -LG denatured and exposed a free thiol (SH) residue. The denatured β -LG could associate with the CN micelle by forming a disulfide-linked complex with κ -CN (1 S-S) located at the surface layer of the CN micelle. β -Lactoglobulin could also interact with other whey proteins possessing Cys residues but to a lesser extent than with the CN micelles, as shown by Vasbinder et al. (2003). Lactoferrin has 17 intramolecular S-S but no free Cys residue. The LF could have undergone thiol/disulfide (SH/S-S) intermolecular exchange reactions with the denatured β -LG and caused the aggregated proteins either to be retained in the rennet curd or to be expelled in the whey fraction upon centrifugation. We suggested that β -LG behaved with LF in a similar manner as with other S-S-containing proteins such as α -LA that possess 4 intramolecular S-S but no free SH. Several studies have shown that upon heating, α -LA intermolecular disulfide reactions are favored in the presence of free SH-containing proteins such as β -LG, but also BSA and ovalbumin (Dalgleish et al., 1997a; Havea et al., 2000; Sun and Hayakawa, 2001; Hong and Creamer, 2002). Differences between skim milk and spiked milk in the concentrations recovered in whey were observed only at 80°C for the more heat-resistant α -LA. The higher stability of α -LA in LF-enriched milk could be ascribed to its structure being stabilized by 4 intramolecular S-S bridges. Dalgleish et al. (1997b) showed that the presence of the free SH containing β -LG was necessary for α -LA to associate with κ -CN upon heating. However, the iron saturation status of LF did not influence the level of β -LG and α -LA recovered in the rennet-spiked milk whey. This result suggests that the binding of iron did not significantly influence the thermal association of LF with the other whey proteins in enriched milk.

Changes in Protein Aggregation in Rennet Curds

To determine the nature of interactions that cause the LF thermal association, 1D and 2D SDS-PAGE analyses were performed on thermally treated skim milk rennet curds and LF-enriched milk. Figure 4A shows the 1D SDS-PAGE pattern in rennet curds of

Figure 4. Nonreduced (NR) and reduced (R) SDS-PAGE of rennet curd from (A) skim milk and (B) milk spiked with LF (0.25% protein, wt/vol) unheated and heated at 60, 70, and 80°C (10 min). C = unheated control; LMW = low molecular weight; HMW = high molecular weight; LF = lactoferrin; Std = standard.

skim milk samples under nonreducing conditions. The first lane corresponds to the unheated control and the subsequent lanes to the heated samples (60, 70, and 80°C). The unheated sample separated into several bands in SDS-PAGE under nonreducing conditions. The major bands were the CN. A band that corresponded to the electrophoretic mobility of LF was resolved above the CN. This LF band was possibly overlapped by another protein or some covalently linked protein oligomers. This band composition was later

characterized by the 2D SDS-PAGE analysis and the presence of dimeric α_{s2} -CN was highlighted (see below). Above the LF + α_{s2} -CN band, a group of slowly migrating bands was observed between the bottom and top ends of the stacking gel. Those bands were, respectively, covalently linked low molecular weight (LMW) and high molecular weight (HMW) aggregates. The intensity of these bands increased with the severity of the heat treatment. This result suggests that covalent protein association occurred upon heating the milk, proba-

bly via thiol/disulfide (SH/S-S) intermolecular reactions. The formation of intermolecular S-S bonds among the whey proteins containing Cys residues (β -LG, α -LA, and BSA) or with κ -CN (1 S-S) or both, but also with α_{s2} -CN (1 S-S), are also known to occur upon heating (Vasbinder et al., 2003; Patel et al., 2006). We previously reported that LF undergo intramolecular S-S cleavages upon heating that initiate aggregation through SH/S-S intermolecular exchange reactions (Brisson et al., 2007). As mentioned above, the presence of LF in covalently linked aggregates has also been observed in heated milk (Nabhan et al., 2004; Patel et al., 2006) and reconstituted skim milk (Jean et al., 2006), suggesting that LF undergoes such reactions with either the whey proteins, the CN, or both.

Similar results were observed in the SDS-PAGE pattern of the milk spiked with native-LF (Figure 4B) as well as with the holo and apo proteins (results not shown). However, the intensity of the overlapped LF band was higher in the case of the curd obtained from the milk spiked with LF. This was probably due to the increased concentration of LF in the enriched milk samples. Moreover, in the nonreduced SDS-PAGE pattern of the LF-enriched milk, the bands related to both the LMW and HMW aggregates were also more intense than those observed in the skim milk. However, the intensity of the band corresponding to, respectively, the LMW and HMW aggregates was lower for the sample heated at 80°C. This result suggested that very large covalently linked aggregates were formed that did not enter the stacking gel pores. These large aggregates were probably loose during either the gel staining or destaining procedure. The decrease in the LF + α_{s2} -CN band intensity was also more important in the thermally treated enriched milk samples. These results suggest that the susceptibility of LF to the formation of intermolecular disulfide-linked aggregates in milk increased with its concentration in milk.

Furthermore, when submitted to SDS-PAGE under reducing conditions, the HMW aggregates observed in the nonreducing SDS-PAGE pattern of both skim milk and the native LF-spiked milk were disrupted into their individual proteins. Apart from the CN, an important band was observed at the bottom end of the resolving gel. This band was identified as para- κ -CN, the insoluble product of the rennet cleavage of κ -CN. This result shows that part of the para- κ -CN, which preserved the 2 Cys residues of the former κ -CN, was covalently linked in the rennet curd. We also observed an increase in intensity of the LF band as the thermal treatment increased. This result also confirmed that the level of LF covalently linked to the CN by intermolecular S-S interactions increased with heating temperatures. The presence of β -LG and, to a lesser extent, α -LA was also

detected at 80°C, suggesting that they were associated in the curd by S-S linkages as well.

A second SDS-PAGE dimension was performed on the 1D SDS-PAGE sample gel strip to identify the proteins that associated into the covalently linked aggregates observed in the first SDS-PAGE dimension. A gel strip from the first SDS-PAGE dimension was removed and reduced prior to the second dimension to cleave the covalently linked aggregates into their respective monomeric proteins, thus allowing their identification. The 2D SDS-PAGE patterns of the unheated and heated (70°C) skim milk rennet curd samples are reported in Figure 5. In the unheated sample 2D SDS-PAGE pattern (Figure 5A), the overlapped LF band from the first SDS-PAGE dimension was reduced to give bands corresponding, respectively in order of electrophoretic mobility, to the monomeric forms of LF and α_{s2} -CN (in dimeric form in the first SDS-PAGE dimension). The presence of α_{s2} -CN dimers has also been reported in milk protein concentrate powder (Havea, 2006) and skim milk (Patel et al., 2006). Patel et al. (2006) also observed that the electrophoretic mobility of α_{s2} -CN dimers in the first SDS-PAGE dimension was very close to LF (overlapping in our experiments). This result shows that a small fraction of LF may have been retained in the rennet unheated milk sample either by noncovalent links with the CN or simply physically entrapment in the curd. The presence of lactoperoxidase, which has a molecular weight of approximately 80 kDa, could not be ruled out. Moreover, the band observed at the bottom end of the stacking gel in the first dimension after reduction resolved as monomeric para- κ -CN in the second dimension. The LMW aggregates observed in the first dimension were reduced in their individual proteins, among which LF, α_{s2} -CN, β -LG, and para- κ -CN were identified in the heated skim milk sample (Figure 5B). All these proteins contained either SH residues (β -LG), S-S bridges (β -LG, LF, α_{s2} -CN, and para- κ -CN), or both. This result suggests that those proteins likely associated upon heating to form large covalently linked copolymers by means of SH/S-S intermolecular exchange reactions. Indeed, the covalently associated LF caused the protein to be retained in the thermally treated milk rennet curd. Although not formally determined, the formation of LF-whey protein coprecipitates expelled in the whey fraction upon centrifugation could not be ruled out. This would also help to explain the lower LF transmission observed in the whey fraction. The amount of HMW aggregates observed in the heated milk in the first dimension was too low to be detected as individual proteins in the second dimension. The heated LF-enriched milk sample (70°C) was also submitted to 2D SDS-PAGE. The gel pattern is shown in Figure 6. The more intense HMW

Figure 5. Two-dimensional SDS-PAGE patterns: nonreduced (NR) SDS-PAGE in the first dimension (horizontally); and reduced (R) SDS-PAGE in the second dimension (vertically) of (A) rennet curd obtained from unheated skim milk, and (B) heated skim milk (70°C, 10 min). 1D = one-dimensional; LMW = low molecular weight; HMW = high molecular weight; LF = lactoferrin.

Figure 6. Two-dimensional SDS-PAGE patterns; nonreduced (NR) SDS-PAGE in the first dimension (horizontally); and reduced (R) SDS-PAGE in the second dimension (vertically) of rennet curd obtained from heated skim milk (70°C, 10 min) spiked with LF (0.25% protein, wt/vol). 1D = one-dimensional; LMW = low molecular weight; HMW = high molecular weight; LF = lactoferrin.

aggregate band observed in the first SDS-PAGE dimension was resolved into the individual proteins in the second reduced SDS-PAGE dimension. As a result, the presence of LF and para- κ -CN, but also a faint band of β -LG, were observed, meaning that they were either self-aggregated or coprecipitated in the rennet-treated enriched milk or both.

CONCLUSIONS

This study showed that thermal treatment can induce the association of LF in rennet-coagulated skim milk and lower its recovery in the corresponding whey fraction. The saturation of LF by iron increases its thermal stability in milk, favoring its recovery in whey. It appears that the thermal association of LF in milk occurred by a combination of noncovalent interactions and also intermolecular SH/S-S exchange reactions for more severe heat treatment. Thermal aggregation of

LF in milk apparently involves both whey proteins and CN possessing Cys residues such as β -LG, α -LA, α _{s2}-CN, and κ -CN.

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