β -Lactoglobulin is a Thermal Marker in Processed Milk as Studied by Electrophoresis and Circular Dichroic Spectra

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ABSTRACT

As much of the sterilization process involves heat treatment during the preparation of milk on an industrial scale, the unpredictable measures of the process are an essential issue in determining the quality of the milk. The purpose of the present study was to investigate the major protein change(s) of whey proteins in processed milk and extend the knowledge for future reference in the dairy industry. Using a native polyacrylamide gel electrophoresis, we showed almost a 90% loss and denaturation of β -lactoglobulin (LG), but not α -lactalbumin (LA), in some brands of the processed and dry milks. Immunochemical analysis using Western blotting revealed that part of the loss was attributed to the formation of large multiple forms of LG in the processed product. Such denaturation was presumably associated with the heating procedure used in the process. Essentially, LG was the only major fraction converted to aggregates in milk heated at 95°C for 30 min on 2-dimensional PAGE. The detailed thermal denaturation of purified LG and LA at various temperatures (50 to 95°C) and time (5 to 960 s) were investigated using a circular dichroic analysis. The maximal changes of ellipticity at 205 nm (converting β -structure to disordered structure) were correlated to heating temperature and time. There were no significant conformational changes of LG at temperatures below 70°C for as long as 480 s. Pronounced and rapid changes occurred between 80 to 95°C in a time-dependent manner. Fifty percent of the maximal changes could be reached within 15 s. In conclusion, the unique chemical and immunochemical loss and conformational changes made LG a superior marker for evaluating the thermal processing of milk. The detailed thermal denaturation curves of LG constructed with its time and temperature in this

study provide a valuable reference for the dairy industry. We postulate that heat treatment over 80°C in 15 s may induce a significant denaturation of milk LG.

(Key words: β -lactoglobulin structure, thermal denaturation, processed milk, α -lactalbumin)

Abbreviation key: CD = circular dichroism, CM = carboxymethylated.

INTRODUCTION

Molten globules are thought to be general intermediates in protein folding and unfolding (Chang et al., 2000; Yang et al., 2001; Croguennec et al., 2004; Song et al., 2005). α -Lactalbumin and LG are 2 of the major protein moieties of bovine whey proteins. Together, they are the most investigated models for understanding the mechanisms involved in protein stability, folding, and unfolding upon heating. Recent studies have shown that milk LA and LG induce apoptosis in tumor cells (Håkansson et al., 1995; Svensson et al., 1999; Casbarra et al., 2004; Baltzer et al., 2004), and produce immunomodulatory (Wong and Watson, 1995; Cross and Gill, 2000; Filteau, 2001; Brix et al., 2003) and hypocholesterolemic effects (Eichholzer and Stahelin, 1993; Nagaoka et al., 2001). More recently, we showed that heating markedly reduced the binding of LG to fatty acid and retinol (Song et al., 2005). Carbonaro et al. (1999) showed that the proteins extracted from cooked common beans were more resistant to proteolysis (due to the formation of protein aggregation) than those from raw beans. The iron absorption of heme from beef exposed to prolonged heating was substantially reduced in humans (Martinez-Torres et al., 1986). Because much of the process involves heat treatment during the preparation of milk on an industrial scale, the unpredictable nature of the process has been an essential issue that may affect the physiologic role of the LA and LG. For this reason, we attempted to investigate some major changes of the whey proteins in the processed milk when heat treatment is mainly involved. In fact, only limited reports have been documented con-

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cerning processed milk thus far. We have reported that a monoclonal antibody prepared against dry milk can specifically recognize dry milk, but not fresh raw milk (Chen et al., 2004). Characterization of this monoclonal antibody revealed that it was directed towards milk LG. Therefore, LG might prove to be a provocative marker in milk quality control. In the present study, we demonstrated that there was a substantial loss of LG in processed milk. Because manufacturers do not disclose the processing procedures of the milk, we established thermal denaturation curves for the loss of LG and LA with respect to their heating times and temperatures. Furthermore, we studied the overall structural changes of LA or LG by circular dichroism and correlated these changes over temperature and time with the loss of LG. Such detailed changes have not been reported previously. The present study provides a reference value for dairy industry preparing high quality products. In addition, Western blot analysis on the changes in some molecular forms of LG in processed milk is also described.

MATERIALS AND METHODS

Preparation of Milk Samples and Whey Protein

Freshly bulked whole raw milk obtained from a local dairy farm was immediately centrifuged at 13,000 rpm ($15,500 \times g$) for 1 h at 4°C. The top layer in the supernatant was carefully removed, and the remaining fraction (whey protein) containing minimal casein was used for PAGE and Western blot. Five brands of commercially processed milk were purchased from the local market and 4 others were from the United States market (Kroger, Cincinnati, OH; Trauth, New Port, KY; United Dairy Farmers, Cincinnati, OH; Weingarden, Loveland, OH). Three powdered milks obtained from the local market were imported from Australia (Klim, Nestle Australia Ltd.), New Zealand (Anchor, NZ Milk Ltd.), and Denmark (Quaker, MD Foods Kjsersing A/S).

Gel Electrophoresis

Sodium dodecyl sulfate-PAGE or native-PAGE containing 15% polyacrylamide was used for the characterization of the whey proteins using a modified procedure similar to that described by Oldfield et al. (1998a). Electrophoresis was conducted in a vertical slab gel unit (Mini PIII, Bio-Rad, Hercules, CA) equipped with a PAC 300 power supply (Bio-Rad). All the samples (8 to 10 μ g) for SDS-PAGE were equilibrated in 10 mM Tris-HCl and 5% SDS, pH 7.6, before loading onto the gel. It is worth noting that preheat treatment for each sample in the conventional SDS-PAGE was omitted to preserve the native structure of milk proteins. The same

procedures were conducted for native-PAGE without the addition of SDS. For 2-dimensional PAGE, 300 μ g of milk protein was first loaded onto the isoelectric focusing gel containing ampholytes (pH 3 to 10) and run for 16 h at 400 V with an additional 1 h at 800 V. The isoelectric focusing gel was then loaded onto a 15% SDS-polyacrylamide slab gel (the second dimension) with a 5% stacking gel as previously established in our laboratory (Yang and Mao, 1999; Wang et al., 2002).

Heat Treatment on Whey Protein Solution Containing LA and LG

To obtain the whey proteins consisting predominantly of LA and LG, whey proteins were fractionated in a final concentration of 40% of saturated ammonium sulfate at 4°C for 30 min. The supernatant was collected by centrifugation at $4000 \times g$ for 30 min at 4°C. The sample was then dialyzed against a Tris-buffered saline containing 50 mM Tris-HCl and 0.12 M NaCl, pH 7.2. Sample solution containing 1 mg/mL of protein was subjected to thermal denaturation at 50, 60, 70, 80, 90, and 95°C, respectively, for 15 to 960 s. The reaction was stopped using a 20°C waterbath.

Heat Treatment on Purified LA and LG

α -Lactalbumin with calcium was purchased from Sigma (lot no. 60k700; St. Louis, MO). β -Lactoglobulin was purified from an HPLC diethylaminoethyl cellulose column in our laboratory using a method similar to that previously described (McCreath et al., 1997). In brief, freshly prepared whey proteins from raw milk were first fractionated using 40% saturated ammonium sulfate. The dialyzed top fraction was then concentrated to protein content at approximately 20 mg/mL. Two milliliters of the solution was applied to a 10×64 mm diethylaminoethyl-5 column (Bio-Rad) and separated using a Waters HPLC system equipped with a 600 controller and 996 photodiode array (Waters, Milford, MA). The sample was then eluted with a 0 to 0.5 M linear NaCl gradient in 0.02 M phosphate buffer, pH 8.0, over 60 min. The flow rate was 1 mL/min. The peak fractions containing LG isoforms were immediately pooled and dialyzed against PBS at 4°C. The heating experiment for LA or LG was then conducted at various temperatures over time according to the procedures mentioned above.

N-Terminal Amino Acid Sequencing

β -Lactoglobulin identified on native-PAGE was sequenced from the N-terminus by an automatic Edman degradation procedure on an ABI 476A peptide se-

Figure 1. Gel electrophoresis of whey proteins obtained from raw and processed milk. Left panel: Native-PAGE. Lane A = raw milk; lane B = processed milk. Right panel: SDS-PAGE. Lane A = raw milk; lane B = processed milk; lane C = purified β -lactoglobulin (LG) and α -lactalbumin (LA) standard. Ten micrograms of the protein sample were loaded onto each lane. There was a marked decrease in 2 acidic proteins in processed milk on the native-PAGE. The acidic proteins were eluted from a transfer blot followed by amino acid sequencing and were subsequently identified as 2 isoforms of LG with first 20 residues as LIVTQTMKGLDIQKVAGTWY. The processed milk was purchased from a local market. M = Molecular weight marker.

quencer (Applied Biosystems, Foster, CA) described previously in our laboratory (Yang and Mao, 1999). Briefly, the protein bands separated on native-PAGE gel were firstly transferred onto a polyvinylidene fluo-

ride membrane. The protein bands corresponding to the expected LG were sliced and subjected to an automatic Edman degradation for amino acid sequence determination.

Figure 2. Native-PAGE analysis of whey proteins obtained from raw, processed, and dry milk. Lane 1 = freshly prepared raw milk (from Taiwan); lanes 2 to 6 = processed milk (5 major brands from local market in Taiwan); lanes 7 to 9 = powdered milk (imported from Denmark, Australia, and New Zealand, respectively); lanes 10 to 13 = processed milk (4 brands from United States). Whey proteins loaded on lanes 1 and 2 were freshly prepared from the same batch, and were obtained from a university dairy farm before and after the sterilization process.

A. Native-PAGE

B. SDS-PAGE

Figure 3. Western blot analysis of whey proteins obtained from raw, processed, and dry milk. Lane 1 = freshly prepared raw milk (from Taiwan); lanes 2 to 6 = processed milk (5 major brands from local market in Taiwan); lanes 7 to 9 = powdered milk (imported from Denmark, Australia, and New Zealand, respectively); lane 10 = typical processed milk (from United States). Notably, the decrease in β -lactoglobulin (LG) was not as sharp as that characterized on Coomassie blue staining due to the extremely high sensitivity of the immunoblot. M = Molecular weight marker.

Carboxymethylation of LG

Approximately 5 mg of LG was first dissolved in 5 mL of 0.1 M Tris-HCl buffer (pH 8.6) containing 6 M

ultrapure urea and 0.02 M dithiothreitol (Song et al., 2005). Following flushing with nitrogen, 20 mg of iodoacetic acid was added into the reaction mixture, the pH was maintained at 8.6 with the addition of 0.1 M

Figure 4. Characterization of heated β -lactoglobulin (LG) using a Western blot analysis on native-PAGE (A) and SDS-PAGE (B). The experiment was carried out by heating isolated LG (1 mg/mL) at 95°C followed by blotting with a polyclonal antibody prepared against LG. Time course is expressed in seconds; there were no aggregates of LG on the initial sample (time 0). C. Chemical modification using carboxymethylation (CM) on all the cysteine residues abolished the formation of LG aggregates. The gel was stained with Coomassie blue. The molecular weight of CM-LG was slightly higher than expected due to the lack of preheating in sample preparation. M = Molecular weight marker.

NaOH, and the mixture was incubated for 3 h at room temperature. Finally, carboxymethylated (CM) LG was desalted on a Bio-Gel P2 column (Bio-Rad) eluted by 0.05 M ammonium bicarbonate and lyophilized. By amino acid analysis, the CM-LG contained 4.9 residues of CM-cysteine per mole of LG.

Circular Dichroism Measurements

For the circular dichroism (CD) measurement, each sample was heated or unheated (0.5 mg/mL) in a Tris-buffered saline containing 50 mM Tris-HCl and 0.12 M NaCl, pH 7.2, was used (Tseng et al., 2004; Song et al., 2005). The CD spectra were conducted on a Jasco-J715 spectropolarimeter (Jasco, Tokyo, Japan) at 24°C over a wavelength range from 200 to 250 nm, and recorded at a scan speed of 20 nm/min. All spectra were measured in a cuvette with a path length of 0.1 cm. Each heated sample (100 μ L) was treated, respectively, at 50, 60, 70, 80, 90, and 95°C. At each temperature, the sample was maintained separately over a period from 15 to 960 s and instantly stopped in a water bath at 20°C before an immediate measurement.

The thermal denaturation curves for LA were constructed according to the formula: percentage of maximal change = [absolute value of ellipticity of 208 nm at each time point/maximal absolute ellipticity of 208 nm at 95°C for 16 min] $\times$ 100. For LG, the maximal absolute value of ellipticity was based on that of 205 nm at 95°C for 8 min.

Preparation of Antiserum Against LG

The polyclonal antibody prepared against LG was raised in female Balb/C mice ($n = 6$) by subcutaneous and intraperitoneal injections of purified LG (Chen et al., 2004). In brief, LG was mixed and homogenized with an equal volume of complete Freund's adjuvant using a 3-way stopcock. Each animal was initially given a total emulsion of 500 μ L containing 200 μ g of LG including 6 subcutaneous injections onto the back and an intraperitoneal injection. After 10 d, 500 μ L of sterile PBS solution containing 200 μ g of LG without adjuvant was boosted by 2 intramuscular injections. Seven days following the final booster, blood was collected in 0.1% EDTA and plasma was obtained. The titer of this antibody was over 1:20,000 as judged by an ELISA using a method previously described (Huang et al., 1999).

Animal Care and Use

Balb/C mice at 5 to 7 weeks of age obtained from National Science Council of Taiwan were fed in animal facility in Chiao Tung University (Hsinchu, Taiwan)

A. Coomassie Blue staining

Raw milk

Heated raw milk

B. Western Blot

Raw milk

Heated raw milk

Figure 5. Two-dimensional gel analysis of raw and heated milk. (A) Coomassie blue staining. (B) Western blot using a β -lactoglobulin (LG) polyclonal antibody. Sample containing 300 μ g of protein was first run in isoelectric focusing (pH 3 to 10 from left to right) followed by a conventional SDS-PAGE.

during the period of immunization. Feed and water were available daily. Mice were sacrificed using CO₂, and other management was conducted according to guidelines established by the National Science Council of Taiwan.

Western Blot

Following the separation of proteins by SDS-PAGE or native-PAGE, the gel was soaked in a buffer con-

taining 50 mM Tris-HCl, 50 mM boric acid, and 1 mM EDTA, pH 8.2, for 30 min (Chen et al., 2004). The gel was then electrotransferred to a nitrocellulose membrane (Hybond-C extra; Amersham, UK) at 100 mA for 1 h in a semidry transfer cell (Bio-Rad). The membrane was immersed in 1% (wt/vol) gelatin for 1 h with gentle shaking. Following a wash with 0.05% Tween 20 containing PBS (pH 7.4) for 3 min, the membrane was incubated with a primary antibody (1:2500 dilution in PBS containing 1% gelatin) for 1 h, and washed 3 times

Figure 6. Native-PAGE analysis of isolated β -lactoglobulin (LG) and α -lactalbumin (LA) (1 mg/mL) heated at 95°C over time. Lane 1 = native LG and LA without heat; lanes 2 to 7 = LG and LA heated over time from 30, 60, 120, 240, 480, and 960 s, respectively. Ten micrograms of each protein were loaded on the native gel.

with PBS containing 1% gelatin. The membrane was then incubated with a diluted second antibody conjugated with horseradish peroxidase against mouse IgG for 1 h in PBS containing 1% gelatin. Finally, the membrane was washed and developed with 3-3'-diaminobenzidine (3, 3', 4, 4'-tetra-amino-biphenyl) containing 0.01% H_2O_2 in PBS.

RESULTS

To identify the possible difference in protein profiles between raw and processed milk, whey proteins from the same batch of the milk before and after the manufacturing process were analyzed on a native-PAGE. Figure 1 (left panel) shows that there was a marked decrease (at least 90%) in 2 acidic proteins of processed milk. N-Terminal sequence analysis revealed that these proteins were the isoforms of LG with a sequence of LIVTQTMKGLDIQKVAGTWY consistent with the chemical characteristics of LG previously reported (Molloy et al., 1977). In the next experiment, we examined the whey proteins on SDS-PAGE using a procedure without heat. Similarly, LG corresponding to molecular weight of about 18 kDa was substantially reduced (Figure 1, right panel). Notably, one extra band corresponding to a molecular weight about 25 kDa in the processed milk was observed on the SDS gel (Figure 1). This protein was not related to LG as assessed by a Western blot (described below), although its biochemical nature remains elusive. We then determined the feature of whey proteins in 5 brands of processed milk from our domestic market. Figure 2 shows that the LG was extensively lost or denatured in those samples (samples 2 to 6). Three brands of dry milk imported from Australia, Denmark, and New Zealand were also analyzed (sam-

ples 7 to 9), in which one brand not only exhibited a large extent of denaturation in LG, but also in LA. However, the LG remained almost intact in 4 randomly chosen brands from the US market (Figure 2). Thus, a simple native-PAGE may be used to impart technological differences involved in processing the milk.

To localize the loss of LG in milk, a polyclonal antibody prepared against native LG was employed for Western blot analysis. The domestic processed milks and imported dry milks (but not the raw milk) revealed large and multiple LG aggregates on native-PAGE (Figure 3A) and SDS-PAGE (Figure 3B). One single band, but no detectable LG aggregates, was observed in raw milk. There were slight and trace aggregates in the US brands.

To test the hypothesis that the electrophoretic change in LG was caused by a heat treatment in the milk processing, we heated the purified LG at 95°C for different times followed by a Western blot. Formation of multiple aggregates of immunoreactive LG was clearly demonstrated on both native- and SDS-PAGE (Figure 4), which was somewhat similar to that in the processed milk (Figure 3). The molecular weight of the smallest LG polymer was about 36 kDa, suggesting that at least a dimer was being formed. It is worth mentioning that because the SDS-PAGE was run without heating, some noncovalently linked LG polymers have existed.

To test whether cysteine residues were responsible for the polymerization upon heating, chemical modification (carboxymethylation) blocking the thio groups of LG was conducted. Figure 4C shows that there was no detectable cross-linking of CM-LG upon heating.

Although we show the substantial loss of LG in processed milk forming large LG polymers, the sum of these polymers did not account for the total loss as shown in Figure 1. The processed milk purchased in the market had usually undergone ultra-heat treatment at 135°C with undisclosed time. In the next experiment, we heated the raw milk at 95°C for 30 min and analyzed its protein profile on 2-D gel electrophoresis. Using Coomassie blue staining, only LG was attenuated; the aggregated polymers were not seen. However, some large molecular forms of LG (either self-associated or other casein-associated) with molecular weight greater than 500 kDa were observed on the Western blot (Figure 5).

We speculated that the initial thermal denaturation of LG was responsible for the cross-linking to form large molecular forms. We studied the structural change of LG caused by heat treatment and compared it with LA, another major milk whey protein. First, the isolated LG and LA were heated at 95°C over time. On native-PAGE analysis, the acidic property of LG was altered in a time-dependent manner (Figure 6). A marked change in heat treatment was observed in LG over time

Figure 7. Effect of temperature and time on the loss of (A) α -lactalbumin (LA) and (B) β -lactoglobulin (LG). The loss of each protein upon the heating was extrapolated from native-PAGE (Figure 6), using the sample without heat as 0% loss. The experiment was conducted independently at various temperatures as indicated.

from 30 s. The major extra band (Figure 6) from the denaturation was LG as confirmed by a Western blot (data not shown). α -Lactalbumin was more resistant (240 s) compared with LG. We further examined the thermal changes in each purified LG or LA at various temperatures over time using the same native-PAGE.

Following the integration using a digital image system from the gel, there was no significant change in LG below 60°C over a period of 960 s (Figure 7). Some moderate changes occurred at 70 to 80°C, but the most pronounced changes occurred above 80°C and were time-dependent. The native form of LG was almost abol-

Figure 8. Effect of temperature and time on circular dichroic spectra of α -lactalbumin (LA) and β -lactoglobulin (LG). (A) LA heated at 50 (—), 60 (---), 70 (·····), 80 (- · - ·), 90 (- · - ·), and 95°C (·····) for 15 s to 15 min. (B) LG heated at 50 (—), 60 (---), 70 (·····), 80 (- · - ·), 90 (- · - ·), and 95°C (·····) for 15 s to 16 min. Notably, the spectrum of unheated was identical to that of heated samples at 50°C for 15 s (data not shown). α -Lactalbumin appears to be more resistant to heat than does LG.

ished when heating proceeded for longer than 240 s. Similarly, the thermal denaturation curves of LA were constructed, but the severity was much less than that of LG in both time and temperature responses (Figure 7B).

Therefore, our data suggested that LG suffered more changes in overall structure than did LA upon heating. To further support this hypothesis, we monitored the structural changes in LG and LA using a CD spectral measurement. Figure 8 shows that the native LG exhib-

Figure 9. Thermal denaturation curves based on the maximal changes of the ellipticity of LA (A) and LG (B) heated at 50 (●), 60 (○), 70 (■), 80 (□), 90 (▲), and 95 (△) °C. The data demonstrate that the transition temperature of LG is between 70 and 80°C. Conformation of LG begins to change above 70°C.

ited primarily a typical β -sheet configuration with a symmetrical deep shape at 215 nm. The β -sheet content is about 50%, consistent with our previous observation (Song et al., 2005). The structure of LG underwent disordered upon continuous heating (50, 60, 70, 80, 90, and 95°C) is shown in Figure 8. Essentially, there were minimal changes in ellipticity when heating between 50 and 70°C over the entire period. Significant structural changes (ellipticity at 205 to 208 nm) were found to occur at and above 80°C. Such changes, however, were not substantial in LA. Extrapolating from the CD studies, thermal denaturation curves for LA and LG were constructed based on the ellipticity (or conformational changes) at each time point vs. the maximal changed ellipticity. Figure 9 shows that there were no or minimal changes at temperatures below 80°C for different time intervals. Marked structural changes were observed when temperatures were above 80°C. Thus, the data suggest that 80°C is a cutoff temperature in maintaining the intact structure of LG.

DISCUSSION

Although the exact physiologic role of milk whey proteins has not been fully elucidated, recent studies have indicated that LA possesses an immunomodulatory activity, conferring increased resistance to the growth of tumors. On the other hand, LG is associated with hypocholesterolemia, transport of retinol (Zsila et al., 2002; Greene et al., 2003; Kontopidis et al., 2004), and antioxidant properties (Salvi et al., 2001; Chevalier et al., 2001; Marshall, 2004). Clinical studies have shown that LG can be directly absorbed into the circulation of infants through the gastrointestinal system (Kuitunen et al., 1994). The immunoreactive LG is recovered 2 h after ingestion of bovine milk. β -Lactoglobulin is therefore an effective vehicle for providing the essential amino acids. Thus, the substantial loss of LG in processed milk found in the present study cannot be ignored. In contrast, the LG of 4 milks from the US market were intact. Our study suggests that the heating procedures used in domestic milk (Taiwan) were significantly different from those in other countries.

Because almost all dairy processes require heat treatments, information on the heat stability of milk with a modified protein component becomes essential. It is conceivable that the dairy industry should be credited for a large number of the knowledge of the biochemical nature of molten globules, as well as improvement in processing whole milk and extending the products used as a nutritional source (Sawyer and Kontopidis, 2000). With respect to the physiologic significance of denatured LG, we have recently shown that LG heated above 80°C produces a marked decrease in fatty acid and

retinol binding (Song et al., 2005). This was due to a significant loss of β -structure within residues 67 to 76 of LG, in which the hydrophobic domain in the pocket of calyx becomes exposed losing its binding nature (Song et al., 2005).

In addition to the nutritional aspects of milk quality, it is certainly important to avoid excessive heating to minimize the formation of deposits in the heating or cooling equipment (De Jong, 1996), which may impair the renneting properties (Lieske, 1997). Detailed knowledge of the denaturation behavior of LG on heating time and temperature is required to promote the positive effects and to minimize the deleterious outcomes (Lieske et al., 1997). In practice, we have found that the deposit of milk remnants did produce damage on the radiators in some facilities of our local dairy manufacturers in Taiwan. Because LG can form clots during the heating process (Bienvenue et al., 2003; Cayot et al., 2003; Guyomarch et al., 2003a; Corredig et al., 2004), overheating of the milk during the sterilization may be responsible for such undesired event.

From the thermal denaturation curves (Figure 7) and CD spectra (Figures 8 and 9), we show that the denaturation of LG was rapid and extensive at 80°C and above. We suggest that the range from 70 to 80°C is a margin. At any treatment above 80°C, intact LG suffers a large loss (or denaturation) undergoes conformational changes. However, the extent of changes was limited in LA. The relatively weak hydrophobic interactions (Chaplin and Lyster, 1986; Ratray and Jelen, 1998) that occur between unfolded LA may explain its high resistance to the thermal changes. The data suggest that the transition temperature of LG occurred between 70 and 80°C consistent with our previous finding using a thermal-sensitive monoclonal antibody as a probe (Chen et al., 2004; Song et al., 2005). It is interesting, however, that prolonged heating (up to 16 min) at transition temperature did not completely denature the LG, with only about 50% loss. Perhaps denaturation and renaturation occur simultaneously and eventually reach equilibrium. A similar case was observed at lower temperatures. At temperatures above the transition, denaturation is not reversible.

Although the denaturation of whey proteins in milk has been studied by native-PAGE (Havea et al., 2002), no information is available on the detailed kinetics of heat-induced conformational changes in LA and LG using CD and native-PAGE simultaneously (50 to 95°C for 15 s to 15 min). The present study provides a detailed and valuable reference for the study of the correlation of conformational changes and electrophoretic properties in both LA and LG.

It is worth mentioning that only the whey proteins, rather than whole milk, were chosen for native-PAGE

in the present study. This is because lipids (micelles) and casein in whole milk greatly affect the performance of gel electrophoresis, as described by others (Xiong et al., 1993; Hollar et al., 1995; Oldfield et al., 1998a,b). Furthermore, because some LG could associate or polymerize with micelle and casein fractions in overheated whole milk (Guyomarc'h et al., 2003a,b; Vasbinder et al., 2003, 2004), this association is responsible for the partial (but not total) loss of LG in whey protein fraction as indicated in our previous study (Chen et al., 2004). Likewise, the immunoreactive LG blotted as in multiple large forms (Figures 3 and 4) may represent the partial loss of total LG in the processed milk. Nevertheless, we demonstrate that the Western blot technique was relatively sensitive in detecting the thermal changes of LG in the milk including its aggregate forms (Chen et al., 2004).

In conclusion, the present study demonstrated a severe denaturation of LG, but not other whey proteins, in local commercially processed and some dry milks. Western blot analysis showed that some of the loss of LG was either self-aggregated or conjugated with other milk proteins. Carboxymethylation of cysteine totally inhibited the LG cross-linking, suggesting that cysteines are responsible for the loss of LG upon the heating. The detailed thermal denaturation curves of LG constructed from the circular dichroic spectra may provide a reference value for the dairy industry in future preparation of milk products. We postulate that heat treatment above 80°C in 15 s may induce a significant loss of milk LG.

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REFERENCES

- Baltzer, A., C. Svanborg, and R. Jaggi. 2004. Apoptotic cell death in the lactating mammary gland is enhanced by a folding variant of alpha-lactalbumin. *Cell Mol. Life Sci.* 61:1221–1228.
- Bienvenue, A., R. Jimenez-Flores, and H. Singh. 2003. Rheological properties of concentrated skim milk: Influence of heat treatment and genetic variants on the changes in viscosity during storage. *J. Agric. Food Chem.* 51:6488–6494.
- Brix, S., L. Bovetto, R. Fritsche, V. Barkholt, and H. Frokiaer. 2003. Immunostimulatory potential of beta-lactoglobulin preparations: Effects caused by endotoxin contamination. *J. Allergy Clin. Immunol.* 112:1216–1222.
- Carbonaro, M., S. Nicoli, and G. Musci. 1999. Heat-induced aggregation of *Phaseolus vulgaris* L. proteins: An electron spin resonance study. *J. Agric. Food Chem.* 47:2188–2192.
- Casbarra, A., L. Birolo, G. Infusini, F. Dal Piaz, M. Svensson, P. Pucci, C. Svanborg, and G. Marino. 2004. Conformational analysis of HAMLET, the folding variant of human alpha-lactalbumin associated with apoptosis. *Protein Sci.* 13:1322–1330.
- Cayot, P., J. F. Fairise, B. Colas, D. Lorient, and G. Brule. 2003. Improvement of rheological properties of firm acid gels by skim milk heating is conserved after stirring. *J. Dairy Res.* 70:423–431.
- Chang, J. Y., A. Bulychiev, and L. Li. 2000. A stabilized molten globule protein. *FEBS Lett.* 487:298–300.
- Chaplin, L. C., and R. L. J. Lyster. 1986. Irreversible heat denaturation of bovine α -lactalbumin. *J. Dairy Res.* 53:249–258.
- Chen, W. L., M. T. Huang, H. C. Liu, C. W. Li, and S. J. T. Mao. 2004. Distinction between dry and raw milk using monoclonal antibodies prepared against dry milk proteins. *J. Dairy Sci.* 87:2720–2729.
- Chevalier, F., J. M. Chobert, C. Genot, and T. Haertle. 2001. Scavenging of free radicals, antimicrobial, and cytotoxic activities of the Maillard reaction products of beta-lactoglobulin glycosylated with several sugars. *J. Agric. Food Chem.* 49:5031–5038.
- Corredig, M., E. Verespej, and D. G. Dalgleish. 2004. Heat-induced changes in the ultrasonic properties of whey proteins. *J. Agric. Food Chem.* 52:4465–4471.
- Croguennec, T., D. Molle, R. Mehra, and S. Bouhallab. 2004. Spectroscopic characterization of heat-induced nonnative beta-lactoglobulin monomers. *Protein Sci.* 13:1340–1346.
- Cross, M. L., and H. S. Gill. 2000. Immunomodulatory properties of milk. *Br. J. Nutr.* 84:S81–S89.
- De Jong, P. 1996. Modeling and optimization of thermal processes in the dairy industry. NIZO Verslag. Ede. V341. NIZO Food Research, the Netherlands.
- Eichholzer, M., and H. Stahelin. 1993. Is there a hypocholesterolemic factor in milk and milk products? *Int. J. Vitam. Nutr. Res.* 63:159–167.
- Filteau, S. M. 2001. Milk components with immunomodulatory potential. *Adv. Nutr. Res.* 10:327–350.
- Greene, L. H., D. Hamada, S. J. Eyles, and K. Brew. 2003. Conserved signature proposed for folding in the lipocalin superfamily. *FEBS Lett.* 553:39–44.
- Guymarc'h, F., A. J. Law, and D. G. Dalgleish. 2003a. Formation of soluble and micelle-bound protein aggregates in heated milk. *J. Agric. Food Chem.* 51:4652–4660.
- Guymarc'h, F., C. Queguiner, A. J. Law, D. S. Horne, and D. G. Dalgleish. 2003b. Role of the soluble and micelle-bound heat-induced protein aggregates on network formation in acid skim milk gels. *J. Agric. Food Chem.* 51:7743–7750.
- Håkansson, A., B. Zhivotovsky, S. Orrenius, H. Sabharwal, and C. Svanborg. 1995. Apoptosis induced by a human milk protein. *Proc. Natl. Acad. Sci. USA* 92:8064–8068.
- Havea, P., H. Singh, and L. K. Creamer. 2002. Heat-induced aggregation of whey proteins: Comparison of cheese WPC with acid WPC and relevance of mineral composition. *J. Agric. Food Chem.* 50:4674–4681.
- Hollar, C. M., N. Parris, A. Hsieh, and K. D. Cockley. 1995. Factors affecting the denaturation and aggregation of whey protein in whey protein concentrate mixtures. *J. Dairy Sci.* 78:260–267.
- Huang, G. S., S. P. Wang, W. T. Chang, T. J. Sun, S. C. Wang, R. Chu, and S. J. T. Mao. 1999. Intracellular processing generated MDA-lys epitope in foam cells. *Life Sci.* 65:285–296.
- Kontopidis, G., C. Holt, and L. Sawyer. 2004. Invited Review: β -Lactoglobulin: Binding properties, structure, and function. *J. Dairy Sci.* 87:785–796.
- Kuitunen, M., E. Savilahti, and A. Sarnesto. 1994. Human alpha-lactalbumin and bovine beta-lactoglobulin absorption in infants. *Allergy* 49:354–360.
- Lieske, B. 1997. The influence of preliminary treatments on structural properties of casein micelles affecting the rennetability. *Lait* 77:201–209.
- Lieske, B., G. Konrad, and W. Faber. 1997. A new spectrophotometric assay for native β -lactoglobulin in raw and processed bovine milk. *Int. Dairy J.* 7:805–812.
- Marshall, K., 2004. Therapeutic applications of whey protein. *Altern. Med. Rev.* 9:136–156.
- Martinez-Torres, C., I. Leets, P. Taylor, J. Ramirez, M. del Valle Camacho, and M. Layrisse. 1986. Heme, ferritin and vegetable iron absorption in humans from meals denatured of heme iron during the cooking of beef. *J. Nutr.* 116:1720–1725.

- McCreath, G. E., R. O. Owen, D. C. Nash, and H. A. Chase. 1997. Preparation and use of ion-exchange chromatographic supports based on perfluoropolymers. *J. Chromatogr. A*. 773:73–83.
- Molloy, M. P., B. R. Herbert, J. X. Yan, K. L. Williams, and A. A. Gooley. 1977. Identification of wallaby milk whey proteins separated by two-dimensional electrophoresis, using amino acid analysis and sequence tagging. *Electrophoresis* 18:1073–1078.
- Nagaoka, S., F. Yu, M. Keiji, A. Takako, Y. Kouhei, K. Yoshihiro, T. Kojima, and K. Tamotsu. 2001. Identification of novel hypocholesterolemic peptides derived from bovine milk β -lactoglobulin. *Biochem. Biophys. Res. Commun.* 281:11–17.
- Oldfield, D. J., H. Singh, and M. W. Taylor. 1998b. Association of β -lactoglobulin and α -lactalbumin with the casein micelles in skim milk heated in an ultra-high temperature plant. *Int. Dairy J.* 8:765–770.
- Oldfield, D. J., H. Singh, M. W. Taylor, and K. N. Pearce. 1998a. Kinetics of denaturation and aggregation of whey protein in skim milk heated in an ultra-high temperature (UHT) plant. *Int. Dairy J.* 8:311–318.
- Ratray, W., and P. Jelen. 1998. Thermal stability of skim milk/whey protein solution blends. *Food Res. Intern.* 30:327–334.
- Salvi, A., P. Carrupt, J. Tillement, and B. Testa. 2001. Structural damage to proteins caused by free radicals: Assessment, protection by antioxidants, and influence of protein binding. *Biochem. Pharmacol.* 61:1237–1242.
- Sawyer, L., and G. Kontopidis. 2000. The core lipocalin, bovine β -lactoglobulin. *Biochim. Biophys. Acta* 1482:136–148.
- Song C. Y., W. L. Chen, M. C. Yang, J. P. Huang, and S. J. T. Mao. 2005. Epitope mapping of a monoclonal antibody specific to bovine dry milk. Involvement of residues 66–76 of strand D in thermal denatured β -lactoglobulin. *J. Biol. Chem.* 280:3574–3582.
- Svensson, M., H. Sabharwal, A. Hakansson, A. K. Mossberg, P. Lipninas, H. Leffler, C. Svanborg, and S. Linse. 1999. Molecular characterization of alpha-lactalbumin folding variants that induce apoptosis in tumor cells. *J. Biol. Chem.* 274:6388–6396.
- Tseng, C. F., C. C. Lin, H. Y. Huang, H. C. Liu, and S. J. T. Mao. 2004. Antioxidant role of human haptoglobin. *Proteomics* 4:2221–2228.
- Vasbinder, A. J., H. S. Rollema, A. Bot, and C. G. de Kruif. 2003. Gelation mechanism of milk as influenced by temperature and pH; studied by the use of transglutaminase cross-linked casein micelles. *J. Dairy Sci.* 86:1556–1563.
- Vasbinder, A. J., F. van de Velde, and C. G. de Kruif. 2004. Gelation of casein-whey protein mixtures. *J. Dairy Sci.* 87:1167–1176.
- Wang, S. H., T. S. Yang, S. M. Lin, M. S. Tsai, S. C. Wu, and S. J. T. Mao. 2002. Expression, characterization, and purification of recombinant porcine lactoferrin in *Pichia pastoris*. *Protein Expr. Purif.* 25:41–49.
- Wong, C. W., and D. L. Watson. 1995. Immunomodulatory effects of dietary whey proteins in mice. *J. Dairy Res.* 62:359–368.
- Xiong, Y. L., K. A. Dawson, and L. Wan. 1993. Thermal aggregation of β -lactoglobulin: Effect pH, ionic environment, and thiol reagent. *J. Dairy Sci.* 76:70–77.
- Yang, J., A. K. Dunker, J. R. Powers, S. Clark, and B. G. Swanson. 2001. Beta-lactoglobulin molten globule induced by high pressure. *J. Agric. Food. Chem.* 49:3236–3243.
- Yang, S. J., and S. J. T. Mao. 1999. A simple HPLC purification procedure for porcine plasma haptoglobin. *J. Chromatogr. B.* 731:395–402.
- Zsila, F., Z. Bikadi, and M. Simonyi. 2002. Retinoic acid binding properties of the lipocalin member beta-lactoglobulin studied by circular dichroism, electronic absorption spectroscopy and molecular modeling methods. *Biochem. Pharmacol.* 64:1651–1660.