Electrophoretic characterization of protein interactions suggesting limited feasibility of accelerated shelf-life testing of ultra-high temperature milk

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ABSTRACT

Accelerated shelf-life testing is applied to a variety of products to estimate keeping quality over a short period of time. The industry has not been successful in applying this approach to ultra-high temperature (UHT) milk because of chemical and physical changes in the milk proteins that take place during processing and storage. We investigated these protein changes, applying accelerated shelf-life principles to UHT milk samples with different fat levels and using native- and sodium dodecyl sulfate-PAGE. Samples of UHT skim and whole milk were stored at 20, 30, 40, and 50°C for 28 d. Irrespective of fat content, UHT treatment had a similar effect on the electrophoretic patterns of milk proteins. At the start of testing, proteins were bonded mainly through disulfide and noncovalent interactions. However, storage at and above 30°C enhanced protein aggregation via covalent interactions. The extent of aggregation appeared to be influenced by fat content; whole milk contained more fat than skim milk, implying aggregation via melted or oxidized fat, or both. Based on reduction in loss in absolute quantity of individual proteins, covalent crosslinking in whole milk was facilitated mainly by products of lipid oxidation and increased access to caseins for crosslinking reactions. Maillard and dehydroalanine products were the main contributors involved in protein changes in skim milk. Protein crosslinking appeared to follow a different pathway at higher temperatures ($\geq 40^\circ\text{C}$) than at lower temperatures, making it very difficult to extrapolate these changes to protein interactions at lower temperatures.

Key words: storage, aggregation, protein interactions, ultra-high temperature, whole and skim milk

INTRODUCTION

Production of UHT milk involves heating the milk to a high temperature (usually 130 to 140°C) for 3 to 5 s, followed by aseptic packaging to produce a commercially sterile product with minimal changes in quality (Holland et al., 2011). Thermal treatment enables storage of UHT milk at room temperature for up to 9 mo, eliminating the need for refrigeration in the distribution chain. Its long shelf life at room temperature has made UHT milk an important food product from nutritional, technological, and economic points of view. However, high temperature treatment induces changes such as whey protein denaturation, Maillard reaction, and mineral imbalances, which may lead to changes in protein–protein interactions and result in physical storage instabilities, including sedimentation of proteinaceous material at the bottom of the storage container, gel formation (age gelation), or both (Andrews and Cheeseman, 1972; McMahon, 1996; Deeth and Lewis, 2016). Proteolysis, another underlining mechanism of gelation, also involves protein network formation (i.e., interaction or linking between proteins or their fragments; Deeth and Lewis, 2016). Irrespective of mechanism, gelation or sedimentation appears to be governed and preceded by changes in the extent and nature of interactions among milk proteins.

Changes in protein interactions primarily involve noncovalent interactions, via either weak bonding (such as hydrophobic, van der Waals, or electrostatic interactions) or covalent crosslinking with other proteins through formation of disulfide bonds, advanced Maillard products (**AMP**), and dehydroalanine (Singh, 1991; Friedman, 1999; Wang et al., 2010; Holland et al., 2011). In addition to protein–protein linking, the role of protein–lipid interactions in the initiation and buildup

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of sediment or gel has not been studied extensively. However, several reports have reported on the lower extent of proteolysis (López-Fandiño et al., 1993; García-Risco et al., 1999), and the creation of products of Maillard reaction (AMP; Valero et al., 2001) in stored UHT milk with higher fat content indicates that fat may play some role in the extent of protein interactions during storage.

Exploring the progression and extent of changes in protein interactions by applying full-length shelf-life tests would be time- and resource-intensive. Accelerated shelf-life tests could be a suitable alternative for predicting storage changes in products such as UHT milk. In fact, by accelerating the rate of deterioration of sensory attributes by exposing the product to elevated storage temperatures, shelf life has been predicted in a comparably short time (Richards et al., 2014). Besides deterioration in sensory attributes, protein-protein interactions are also enhanced at higher storage temperatures (Al-Saadi and Deeth, 2008). Therefore, using elevated storage temperatures, protein interactions could also be accelerated, and the results could then be extrapolated to predict aggregation mechanisms at lower storage temperatures using a rapid method of determination such as Fourier transform infrared spectroscopy, avoiding lengthy real-time analysis. However, as has been previously reported, higher storage temperatures ($\geq 40^{\circ}\text{C}$) inhibit age gelation (Kocak and Zadow, 1985; McMahon, 1996; Deeth and Lewis, 2016). The reasons for this are not known, but a few suggestions have been postulated. First, an increased rate of proteolysis at higher temperatures results in extensively degraded proteins, which are no longer able to form a stable gel matrix (Datta and Deeth, 2001). The second hypothesis is that accelerated Maillard browning reactions at higher temperatures block lysine residues, which would likely participate in protein-protein interactions leading to gelation (Samel et al., 1971; Gaucher et al., 2008). Third, higher rates of chemical crosslinking via Maillard and other reactions prevents the release of $\beta\kappa$ complex from the micelle, which is the starting point for the onset of age gelation (McMahon, 1996). Furthermore, high storage temperatures slow gelation but result in increased nondispersible sediment (Nieuwenhuise and van Boekel, 2003). Thus, accelerated shelf-life testing in UHT milk could only be used to predict sedimentation at room temperature.

The present study was aimed at establishing the feasibility of using elevated temperatures during storage of UHT milk with different fat contents to predict changes in the interactions of milk proteins that may lead to sedimentation at room temperature.

MATERIALS AND METHODS

Materials

Commercial UHT whole (full cream) milk (**WM**) and skim milk (**SM**) were provided by a local manufacturer (Murray Goulburn Co-operative Co. Ltd., Victoria, Australia). All milk packs originated from the same batches. Both SM and WM packs were produced on the same day using an established process on an indirect tubular processor (SPX Flow Technology, Mulgrave, Australia) with a 9,000 L/h capacity at 138°C for 6 s. The composition of the WM as reported by the manufacturer was 33 g/L protein, 34 g/L fat, 53 g/L sugars, 0.55 g/L sodium, and 1.2 g/L calcium. The composition of the SM was 34 g/L protein, 1 g/L fat, 53 g/L sugars, 0.55 g/L sodium, and 1.2 g/L calcium.

The electrophoresis chemicals were obtained from Bio-Rad Laboratories (Richmond, CA). The reducing agent 2-mercaptoethanol, bovine milk standards α -LA, β -LG, and BSA were purchased from Sigma (Castle Hill, NSW, Australia). Prestained SDS-PAGE standard (SeeBlue Plus2) was procured from Thermo Fisher Scientific (Scoresby, Victoria, Australia).

Storage of UHT Milk

The UHT milk packs were stored at room temperature (20°C) and 3 elevated temperatures (30, 40, and 50°C) for 28 d in incubators (Thermoline Scientific Pty Ltd., Wetherill Park, NSW, Australia). The elevated storage temperature served to accelerate the development of storage instabilities. Milk packs were analyzed on the first day of delivery (d 0), and then at two 2-wk intervals (14 and 28 d) during storage for change in the interactions of milk proteins.

Electrophoretic Analysis of Changes in Protein Interactions

We investigated changes in the interactions of milk proteins during storage of UHT milk using gel electrophoresis—native- and SDS-PAGE under reducing and nonreducing conditions, as previously described (Disanayake et al., 2013), with some minor modifications. For native-PAGE, milk from each pack was diluted with the native sample buffer [0.11 M Tris-HCl buffer (pH 6.8), 8.8% (vol/vol) glycerol, 2.22% (vol/vol) of 0.4% (wt/vol) bromophenol blue solution]. A working volume of 10 μL was then loaded on 12.5% gels and run for 85 min at 210 V and 70 mA in a Protean II xi cell (Bio-Rad Laboratories), filled with tank buffer solu-

tion (0.025 *M* Tris, 0.19 *M* glycine, pH 8.3). Gels were rinsed in MilliQ water for 15 min and stained using staining solution (0.15% Coomassie Brilliant Blue R250 dye, 72% isopropanol, and 3% acetic acid) by slowly shaking for 1 h. The stain was completely removed, and the gel was destained by shaking it slowly in destaining solution (10% isopropanol, 10% acetic acid) overnight on a shaking platform.

The same protocol used for native-PAGE was followed for SDS-PAGE, except that we used an SDS sample buffer [0.0625 *M* Tris-HCl buffer (pH 6.8), 10% (vol/vol) glycerol, 2.5% (vol/vol) of 0.4% (wt/vol) bromophenol blue solution, 20% (vol/vol) of 10% (wt/vol) SDS]. In reducing SDS-PAGE, 20 μ L of 2-mercaptoethanol was added per 1 mL of milk and SDS buffer mix, vortexed,

heated in a boiling water bath for 5 min, and cooled before running on SDS-PAGE gels in SDS running buffer (0.1% SDS, 0.025 *M* Tris, and 0.191 *M* glycine, pH 8.6). We used prestained SDS-PAGE standard (3–200 kDa) to compare molecular weights. Milk samples from different packs were analyzed in duplicate. Representative gel patterns are shown in Figures 1 to 3. Native-PAGE and SDS-PAGE gels were scanned using a ChemiDoc imager (ChemiDoc MP; Bio-Rad Laboratories).

Quantitation and Kinetics of Changes in Protein Interactions

We determined the intensities of the major protein bands in reducing SDS-PAGE using the Image Lab

Figure 1. Native-PAGE patterns of UHT skim milk and whole milk on d 0, 14, and 28 of storage. Lanes labeled L1, L2, L3, and L4 represent protein standards of β -LG, α -LA, calcium caseinate, and BSA on d 0, respectively. L5 represents controls: skim milk (A) and whole milk (D). The numbers 20, 30, 40, and 50 indicate samples stored at 20, 30, 40, and 50°C at a given storage time for skim milk (A, B, and C) and whole milk (D, E, and F). Circled lanes indicate accelerated band fading. Color version available online.

5.2.1 software associated with the densitometer (Bio-Rad Chemidoc MP imaging system). We calculated the absolute quantity of reduced proteins from their respective band intensity, and because the gels were run in duplicate, the absolute quantity was averaged. The average loss in absolute quantity of reduced milk proteins and increase in amount of aggregates obtained at different storage temperature was plotted versus time.

Statistical Analysis

The experiments were arranged in a block, split plot in time design, with fat content as the main factor and time as a subplot. The design was replicated on a separate occasion, which served as a block. All samples were also subsampled at least twice, giving at least 4 independent observations. The data were analyzed using the GLM procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC). The level of significance was preset at $P < 0.05$.

RESULTS AND DISCUSSION

Changes in protein interactions during the storage of UHT milk under different conditions were demonstrated by alterations in electrophoretic mobility. We explored the type and extent of the interactions at room temperature and at elevated temperatures using native, nonreducing, and reducing SDS-PAGE assays. We identified different bands in patterns by concurrent electrophoresis of standards and by comparing the results with similar reported patterns in the literature (Patel et al., 2007).

Effect of Storage Conditions on Loss of Native Proteins

The effect of storage time and temperature on native-PAGE patterns in SM and WM is presented in Figure 1. The patterns show thick bands at the entry of stacking gel, corresponding to high-molecular-mass protein aggregates, which were not able to penetrate the gel.

Figure 2. Nonreducing SDS-PAGE patterns of UHT skim milk (SM) and whole milk (WM) on d 0 (control), 14, and 28 of storage. Lanes labeled L1, L2, L3, L4, and L5 represent a molecular weight marker and protein standards for α -LA, β -LG, calcium caseinate, and BSA, respectively. Skim milk and whole milk samples were stored at d 0, and 20, 30, 40, and 50 denote samples stored at 20, 30, 40, and 50°C at a given storage time. Circled lanes indicate accelerated fading and aggregation. Color version available online.

Figure 3. Reducing SDS-PAGE patterns of UHT skim milk (SM) and whole milk (WM) on d 0, 14, and 28 of storage. Storage samples were reduced with β -mercaptoethanol and electrophoresed on SDS gels (4–12.5%). Lanes labeled L1, L2, L3, L4, L5, and C represent a molecular weight marker and protein standards for α -LA, β -LG, calcium caseinate, BSA, and SM and WM samples stored at 0 d, respectively. The SM and WM samples were stored at d 0, and 20, 30, 40, and 50 denote samples stored at 20, 30, 40, and 50°C at a given storage time. Circled lanes indicate accelerated fading and aggregation. Color version available online.

Aggregates probably corresponded to denatured whey proteins associated with themselves and with caseins via thiol-disulfide exchange (Guyomarc'h et al., 2003; Anema, 2008), noncovalent interactions (Donato and Guyomarc'h, 2009), or covalent interactions (Al-Saadi et al., 2013). Faint bands corresponding to native-like whey proteins—namely, α -LA and β -LG, further supported this suggestion. Patterns also exhibited bands corresponding to native κ -CN, β -CN, and α_{S1} -CN. We observed a diffusely stained region between κ -CN and β -CN bands, which has been attributed previously to crosslinked milk proteins (Patel et al., 2007). Native-PAGE patterns in both SM and WM at the start of storage (d 0) appeared to be similar (Figures 1A and D). However, we observed more prominent changes between these samples during storage, depending on the storage temperature.

Storage of SM and WM at 20, 30, or 40°C for 14 d did not alter the intensity of the bands corresponding to individual native-like caseins (Figures 1B and

1E). However, after 28 d of storage at the same temperatures, the intensity of all native protein bands appeared to decrease for both SM and WM (Figures 1C and F). Notably, loss in “native-like” band intensities of individual milk proteins appeared to be higher in WM at 40°C after 28 d than in SM. This could be due to liquefaction of milk fat around 40°C (MacGibbon and Taylor, 2006). Fat globules are surrounded by caseins and whey proteins upon homogenization, and when melted, they may act as a “glue” between adjacent proteins, increasing the proteins’ chance of interaction. A similar observation has been made for the lower heat stability of homogenized milk (Huppertz, 2016) because of the predominant presence of a casein fraction on homogenized fat globules, increasing a volume fraction of casein micelles and enhancing casein particles’ rate of encounter, leading to enhanced heat-induced coagulation. Another proposed mechanism driving these changes is oxidation of lipids at elevated storage temperatures. The intermediates (e.g., free

radicals and hydroperoxides) and end products (e.g., malondialdehyde) from lipid peroxidation interact with AA residues such as cysteine, lysine, histidine, valine, methionine, and phenylalanine, resulting in protein crosslinks (Singh, 1991; Le et al., 2011). In addition to changes in the physical state of lipids and greater generation of the products of lipid oxidation, promoting protein aggregation at higher temperatures, other bulk physiochemical changes (including decline in pH and increase in ionic calcium activity) could also have contributed to the observed differences in native-PAGE patterns for SM and WM (Boumpa et al., 2008). The effect of fat content on ionic calcium activity and its subsequent effect on protein interactions during storage is not known at present and requires further elaboration.

Although only WM indicated accelerated aggregation of milk proteins at 40°C, native-PAGE patterns for both SM and WM samples stored at 50°C were substantially different from those at other temperatures. After 14 d of storage at 50°C in both SM and WM, bands corresponding to β -LG and α -LA completely disappeared, with a concomitant decrease in intensity of α_{S1} -CN, κ -CN, and β -CN bands (Figures 1B and E). The intensity of the diffuse region between κ -CN and β -CN bands also diminished. The complete disappearance of bands corresponding to β -LG and α -LA indicated greater reactivity of whey proteins in the formation of aggregates. This was likely caused by denaturation of β -LG and α -LA and exposure of lysine residues to a high level in these proteins. These characteristics render them a preferable reactant for lactosylation over caseins (Le et al., 2011). Lactosylated whey proteins may further be converted to AMP at higher storage temperatures, which then also crosslink caseins and result in loss of native-like caseins (Le et al., 2011). Further fading out of bands occurred after 28 d of storage, indicating even greater crosslinking of proteins and at a far higher rate than at other storage temperatures in both SM and WM (Figure 1C and F). As well as increasing concentrations of AMP, higher storage temperatures also accelerate other protein crosslinks through intermediates and end products of lipid oxidation (predominantly in WM), dehydroalanine and hydrophobic or other noncovalent interactions (Singh, 1991; Holland et al., 2011). Dehydroalanine is produced by heat-induced elimination of phosphate from phosphoserine residues in caseins. It then reacts with the amino group of lysine residues, the imidazole group of histidine, or the thiol group of cysteine, resulting in intra- or intermolecular lysinoalanine, histidinoalanine, or lanthionine crosslinks, respectively (O'Connell and Fox, 2003). Higher storage temperature also induces changes in the secondary structure of

milk proteins (Mahler et al., 2009). This results in the exposure of hydrophobic regions, which may associate via noncovalent interactions (Andrews, 1975; Henle et al., 1996; Donato and Guyomarc'h, 2009; Holland et al., 2011).

Effect of Storage Temperature on Loss of SDS Monomeric Proteins

To obtain information regarding the type of aggregation (covalent and noncovalent) and the involvement of particular milk proteins, we further analyzed milk samples using the dissociating agent SDS without the reducing agent. We know that SDS dissociates intermolecular and intramolecular aggregates that are noncovalently linked. Thus, nonreducing SDS-PAGE could reveal the involvement of hydrophobic interactions in the formation of the high-molecular-mass aggregates observed in native-PAGE patterns.

Figure 2 shows SDS-PAGE nonreducing patterns for both SM and WM. These patterns showed intense bands corresponding to monomeric α_{S1} -CN, κ -CN, and β -CN, α_{S2} -CN dimer and BSA. Bands corresponding to SDS monomeric α -LA and β -LG were faint but had a higher intensity than native bands, implying that these proteins were linked via noncovalent interactions in addition to disulfide or other covalent bonds (Havea et al., 1998; Donato and Guyomarc'h, 2009). Reduction in the intensity of high-molecular-mass aggregates at the top of the stacking gel further supported this suggestion (Figures 1 and 2). However, noticeable bands corresponding to high-molecular-mass aggregates and diffuse regions at the top of the stacking gel and resolving gel, respectively (Figure 2), indicated that these aggregates were either disulfide-linked or covalently bonded. Furthermore, SDS-PAGE patterns of SM and WM at the start of storage were similar (Figure 2), suggesting that the effect of UHT treatment was independent of fat content. This finding contradicted the theory that milk fat protects other components of milk from heat-induced changes (Pellegrino, 1994). However, the effect of fat content appeared to be more relevant during storage at different temperatures.

At and below 40°C, storage for the first 14 d did not change the intensity of protein bands in SM (Figure 2). However, in WM, the intensity of bands corresponding to whey proteins decreased, with a concomitant increase in the intensity of aggregate bands at the top of the resolving gel. This was in line with similar observations made in native-PAGE patterns of WM after 14 d of storage at 40°C, implying the likely involvement of fat. After 28 d of storage for SM and WM at 20, 30, and 40°C, we observed a decrease in the intensity

of different SDS monomeric bands (Figure 2). Thus, covalent crosslinking of proteins progressed at a slower rate, even at lower storage temperatures (Gaucher et al., 2008). However, in WM, the bands corresponding to whey proteins were diffuse and faint compared with SM at the same temperature. The decrease in band intensity of whey proteins was further exacerbated in WM at 50°C. After 28 d of storage at this temperature, whey protein bands disappeared, and we were able to observe only a diffuse region (smear) with very faint bands corresponding to high-molecular-mass protein aggregates α_{S1} -CN and β -CN in WM (Figure 2). This diffuse region has been attributed to nondisulfide covalent crosslinking of proteins (Singh, 1991). Skim milk also exhibited an intense aggregate band at the top of the resolving gel and faint bands corresponding to β -CN, α_{S1} -CN, and whey proteins at 50°C. The faster and more complete disappearance of whey proteins in WM at and above 40°C could be due partly to enhanced covalent crosslinking stemming from changes in the physical state of fat and the oxidation of lipids. Also, other unexplored mechanisms involving changes in pH or mineral balance differing affected by the presence of fat might have resulted in the different electrophoretic patterns observed for WM.

Only α_{S1} -CN and β -CN were present in both SM and WM in SDS-PAGE nonreduced at 50°C after 28 d; this could be due to their comparatively higher amounts in milk (Huppertz, 2013). Furthermore, because of their more hydrophobic nature and lack of cysteine residues, α_{S1} -CN and β -CN may be more prone to involvement in noncovalent interactions than other caseins.

Effect of Storage Conditions on Loss of Reduced Proteins

We further explored the nature of the aggregates and smear (disulfide or other type of covalently bonded) produced at higher temperatures in SDS-PAGE non-reducing patterns by using SDS-PAGE under reducing conditions. The SDS dissociated hydrophobic interactions and, with the addition of β -mercaptoethanol, it reduced disulfide bonds. Hence, the bands corresponding to aggregates in SDS-PAGE reducing patterns would be entirely due to nondisulfide covalent interactions.

Skim milk and WM had similar patterns on d 0 for native-PAGE and SDS-PAGE nonreducing patterns (Figures 1 to 3). This finding reconfirmed previous observations that the effect of UHT treatment on the electrophoretic mobility of milk proteins was independent of fat content. At the beginning of storage, high-molecular-mass aggregates were not present on top of the stacking gel (Figure 3). Only a faint band

demonstrative of nonreducible protein aggregates were present at the top of the resolving gel, as shown earlier for nonreducing SDS-PAGE. Furthermore, patterns on d 0 had intense bands corresponding to caseins, β -LG and α -LA, suggesting that thiol-disulfide exchange and hydrophobic interactions were major aggregation pathways at the beginning of storage (Pizzano et al., 2012). We also observed a diffuse region between the nonreducible aggregate band and the α_{S2} -CN dimer, and between BSA and the α_{S2} -CN dimer. This region probably also had nondisulfide, covalently linked caseins and whey proteins, because it was present in native-PAGE and in nonreducing SDS-PAGE patterns. It appeared that at the beginning of storage, the order of interactions in both SM and WM was disulfide > noncovalent > covalent (nondisulfide). Although SM and WM had similar patterns on d 0, storage conditions affected them differently in the context of extent of aggregation and loss of different individual milk proteins.

Kinetics of Aggregate Formation

Skim milk did not exhibit an increase in the amount of nonreducible and high-molecular-mass aggregates after 28 d of storage at 20°C (Figures 3 and 4). However, after 28 d of storage at 30 and 40°C, we did note an increase in nonreducible aggregates, although not in high-molecular-mass aggregates. In WM, the trend was slightly different: the nonreducible aggregates started to build up only after 14 d of storage at 20, 30, or 40°C. However, like SM, WM showed no apparent increase in the concentration of high-molecular-mass aggregates, even after 28 d of storage (Figures 3 and 6). At 50°C, the amount of nonreducible and high-molecular-mass aggregates increased to a great extent in both SM and WM (Figure 4). The proteins were most likely aggregated by covalent interactions to form high-molecular-mass aggregates, and rate of these interactions was enhanced sharply above 40°C. Furthermore, the extent of formation of high-molecular-mass aggregates was greater in WM than in SM following 28 d of storage (Figure 4), suggesting enhanced protein aggregation at higher temperatures in the presence of fat.

Besides the difference in the amounts of aggregates formed, SM and WM also differed by how individual caseins and whey proteins participated in the formation of covalently bound aggregates. In SM at 20°C, we observed no change in the quantity of whey proteins and caseins (Figure 5A), supporting our earlier observation that there was no change in reducible and high-molecular-mass aggregates (Figures 3, 4A). In SM at 30 and 40°C, α_{S2} -CN, β -CN, and β -LG decreased during the first 14 d of storage, followed by α_{S2} -CN

and α -LA during the next 14 d of storage (Figures 5B and C). However, in WM, the reduction in the quantity of proteins started at 20°C (Figures 6A to C). At 20, 30, and 40°C, β -LG was one of the first proteins to initiate nondisulfide aggregation in WM, as evidenced by its decrease in concentration after the first 14 d of storage (Figures 6A to C). A reduction in the quantity of α_{S1} -CN, α_{S2} -CN, and β -LG in WM was evident over the next 14 d at 20 and 30°C (Figures 6 A and B). At 40°C, α -LA also participated in aggregation through nondisulfide covalent interactions in WM, similar to SM. Loss in the quantity of proteins was much higher at 50°C than at 20, 30, or 40°C in both SM and WM. In addition, κ -CN participated in covalent aggregation only in the latter half of storage at 50°C in both WM and SM, overruling proteolysis by bacterial proteinases.

All the proteins in milk seemed to be actively involved in crosslinking at 50°C. Higher storage temperatures increase intra-micellar crosslinking, which might result in increased electrostatic repulsion inside the micelle and lead to dissociation and sedimentation of cross-linked proteins (Nieuwenhuise and van Boekel, 2003). However, β -CN and α_{S2} -CN were main players in SM, whereas α_{S1} -CN, α_{S2} -CN, and α -LA were central to protein-protein interactions in WM (Figures 5 and 6). The difference in type of caseins involved in WM and SM at different temperatures suggested preferences for specific pathways of covalent (nondisulfide) crosslinking.

Nondisulfide covalent crosslinking in UHT milk during storage occurs via AMP, dehydroalanine, and lipid oxidation products (in WM) (Singh, 1991; Le et al., 2011). Crosslinking via AMP involves lactosylation of

Figure 4. Change in absolute quantity of nonreducible (A and B) and high-molecular-mass (C and D) aggregates during storage of UHT skim milk (A, C) and whole milk (B, D) at storage temperatures of 20°C (●), 30°C (▲), 40°C (◆), and 50°C (■), as obtained from image analysis of reducing SDS-PAGE. Error bars represent SD.

Figure 5. Change in absolute quantity of caseins (α_{S1} -CN, ●; α_{S2} -CN, ■; β -CN, ◆; κ -CN, ○) and whey proteins (β -LG, ▼; α -LA, ▲) during storage of UHT skim milk at storage temperatures of 20°C (A), 30°C (B), 40°C (C), and 50°C (D), as obtained from image analysis of reducing SDS-PAGE. Error bars represent SD.

lysine residues as the first step (Van Boekel, 1998), implying that proteins with a higher number of lysine residues would be preferentially involved in this reaction. The lysine content of different milk proteins vary, following this order: α_{S2} -CN > β -LG > α_{S1} -CN > α -LA > β -CN > κ -CN (Huppertz, 2013). Involvement of α_{S2} -CN as one of the major players in covalent aggregation in SM (Figure 5) indicates higher levels of AMP-mediated crosslinking. Additionally, disappearance of α -LA in SM even at 30°C also suggests a higher rate of Maillard reaction in the former. The preference of α -LA for reaction with lactose was demonstrated earlier by lactosylation of almost all its lysine residues during storage of UHT milk (Holland et al., 2011). Moreover, α -LA may also preferentially engage in this reaction (Le et al., 2011) because of conformational changes and

denaturation that take place during UHT treatment (Losito et al., 2010). The greater extent of Maillard reaction in SM is also supported by the comparatively higher increase in the “b” value of color in SM than WM at temperatures above 30°C (data not shown). Valero et al. (2001) had also reported higher levels of Maillard products in SM than WM during storage. Greater AMP-mediated crosslinking in SM also explained the reduction in quantity of β -CN in SM. Lactosylation results in increased hydrophilicity of the casein micelle interior, which might have weakened hydrophobic associations (Rauh et al., 2015) and released β -CN to the solution (Figure 7A). In addition to lactosylation, α_{S2} -CN also appears to be most reactive candidate for dehydroalanine-linked proteins, because of a high number of phosphoserine residues and the presence of 2

cysteine residues (O'Mahony and Fox, 2013). Skim milk with greater involvement of α_{S2} -CN and β -CN implied a higher rate of AMP and dehydroalanine-mediated crosslinking (Figure 7A).

In WM, α_{S1} -CN, α_{S2} -CN, and α -LA were the main proteins involved in nondisulfide covalent crosslinking (Figure 6). Previous reports have shown α_{S1} -CN to be the most abundant protein in aggregates (Holland et al., 2011). Furthermore, the higher amount of aggregates in WM than in SM can be attributed to crosslinking via lipid oxidation products and melted fat globules, at and above 40°C in WM. The modified fat globule membrane also consists of whey proteins and predominantly caseins besides its native components (Huppertz, 2016). In addition to change in state and

oxidation of milk fat, high storage temperatures lower pH and increase ionic calcium activity during storage of UHT milk (data not shown). Homogenized fat globules are stabilized by casein micelles on their surface, which may not contain enough κ -casein molecules on the outside due to distorted and randomized spreading (Huppertz, 2016). As all other caseins are calcium-sensitive, protein coagulation and thus aggregation in WM would be enhanced due to the increase in ionic calcium activity (Huppertz, 2016). Therefore, the difference in involvement of different proteins in SM and WM might be due to differences in their access and availability. Thus, it appeared that covalent crosslinking in UHT WM was facilitated via products of lipid oxidation and increased access to caseins for crosslinking reactions,

Figure 6. Change in absolute quantity of caseins (α_{S1} -CN, ●; α_{S2} -CN, ■; β -CN, ◆; κ -CN, ○) and whey proteins (β -LG, ▼; α -LA, ▲) during storage of UHT whole milk at storage temperatures of 20°C (A), 30°C (B), 40°C (C), and 50°C (D), as obtained from image analysis of reducing SDS-PAGE. Error bars represent SD.

Figure 7. Advanced Maillard products (AMP), dehydroalanine (DHA), and lipid oxidation products mediated protein crosslinking in (A) skim milk (SM) at and above 30°C and (B) whole milk (WM) at and above 40°C . AWP = aggregated whey proteins; CCP = colloidal calcium phosphate. Color version available online.

and that Maillard and dehydroalanine products were the main contributors for the observed changes in UHT SM (Figure 7B). However, these suggestions would require further elaboration.

In conclusion, protein interactions—especially covalent crosslinking—were accelerated at higher storage temperatures. However, at temperatures above 30°C in WM and above 40°C in SM, the rate and type of these reactions were very different from those at 20°C. Especially in case of WM, protein crosslinking appeared to follow a different pathway that may not occur in storage at lower temperatures. In addition, the pathways discussed above that enhance protein interactions were only the major ones and did not include all possible factors, which are numerous because of the complex nature of milk. Extrapolating these reactions to predict changes in protein interactions at lower temperatures is very difficult and emphasizes the need for long-term study. However, acquired knowledge of the involvement of individual proteins and the kinetics of these changes may assist in identifying structural changes that can be determined by a spectral analysis, such as Fourier transform infrared spectroscopy. A detailed long-term study would also need to be undertaken to confirm these changes and establish them as markers for prediction of the shelf life of UHT milk using spectral analysis.

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