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Proteomic Analysis of Temperature-Dependent Changes in Stored UHT Milk

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Supporting Information

ABSTRACT: Molecular changes in milk proteins during storage of UHT-treated milk have been investigated using two-dimensional electrophoresis (2-DE) coupled to MALDI-TOF mass spectrometry. UHT-treated samples were stored at three different temperatures, 4 °C, 28 °C, and 40 °C, for two months. Three main changes could be observed on 2-DE gels following storage. They were (1) the appearance of diffuse staining regions above the position of the monomeric caseins caused by nondisulfide cross-linking of α and β-caseins; (2) the appearance of additional acidic forms of proteins, predominantly of α_{S1}-casein, caused by deamidation; and (3) the appearance of "stacked spots" caused by lactosylation of whey proteins. The extent of the changes increased with increased storage temperature. Mass spectrometric analysis of in-gel tryptic digests showed that the cross-linked proteins were dominated by α_{S1}-casein, but a heterogeneous population of cross-linked forms with α_{S2}-casein and β-casein was also observed. Tandem MS analysis was used to confirm deamidation of N^{129} in α_{S1}-casein. MS analysis of the stacked spots revealed lactosylation of 9/15 lysines in β-lactoglobulin and 8/12 lysines in α-lactalbumin. More extensive analysis will be required to confirm the nature of the cross-links and additional deamidation sites in α_{S1}-casein as the highly phosphorylated nature of the caseins makes them challenging prospects for MS analysis.

KEYWORDS: two-dimensional electrophoresis, cross-linking, deamidation, lactosylation, casein, whey, MALDI-TOF MS

INTRODUCTION

Thermal treatment of milk is used to reduce bacterial load and thereby extend shelf life. Pasteurization typically involves treatment at 72 °C for 15 s and produces a pathogen-free but nonsterile product with a shelf life of about 14 days at 4 °C. Ultrahigh temperature (UHT) treatment involves heating to 130−140 °C for 3−5 s and produces an effectively sterile product with a shelf life of many months at ambient temperature.[1]

A phenomenon which reduces shelf life is age gelation. Several factors are known to influence the time of onset of gelation, with storage temperature being a major one.[2] Gelation occurs earliest at ∼25−30 °C but is delayed at higher and lower temperatures.[3]

The heating process induces a number of chemical reactions which can be exacerbated by subsequent storage at elevated temperatures. The best known of these is the Maillard reaction, which involves predominantly the reaction between reducing sugars (i.e., lactose) and the free amine group of protein-bound lysine residues.[4−7] The initial lactosylation can be followed by a complex series of reactions, which depend on the severity and duration of heating.

Protein cross-linking is another common feature of heated milk. Disulfide bond formation between β-lactoglobulin (β-Lg) and kappa-casein occurs following denaturation of β-Lg, which exposes a free sulfhydryl group leading to both intramolecular and intermolecular thiol−disulfide exchange reactions.[8] Additional cross-linking can occur via the formation of dehydroalanine.[9] The most likely mechanism of this is heat-induced β-elimination of phosphate from the phosphoserine residues in caseins. Subsequent reaction of dehydroalanine with the ε-amino group of lysine residues produces intra- or intermolecular lysinoalanine cross-links. Dehydroalanine can also react with histidine or cysteine to produce histidinoalanine or lanthionine cross-links.

These heat-induced changes can have deleterious effects on the nutritional and functional properties of milk proteins. Lactosylation reduces the level of available lysine, which may decrease the nutritional value of milk or milk products. Protein cross-linking can lead to the formation of aggregates and insoluble precipitates, which also reduce the quality and functionality of milk and milk products. Consequently, these heat-induced changes have been of considerable interest to the dairy industry for many years.

Increasingly, mass spectrometric approaches based on LC−MS or LC−MSMS are being used to study the bovine milk proteome and to monitor milk quality.[10−12] The Maillard reaction in particular has been extensively examined by LC−MS approaches.[7,13−24] Proteomic technologies based on two-dimensional electrophoresis (2-DE) coupled to MS have been used both for global analysis of bovine milk proteins and to examine individual proteins in detail.[19,25−37] A combination of reducing/nonreducing 2-DE has been used to examine native disulfide bonding patterns[38] and more recently to examine disulfide cross-linking in milk heated for up to 30 min.[39]

In this work we have used 2-DE coupled with MALDI-TOF MS to examine the effects of storage at elevated temperature on UHT milk. This approach allowed simultaneous demonstration

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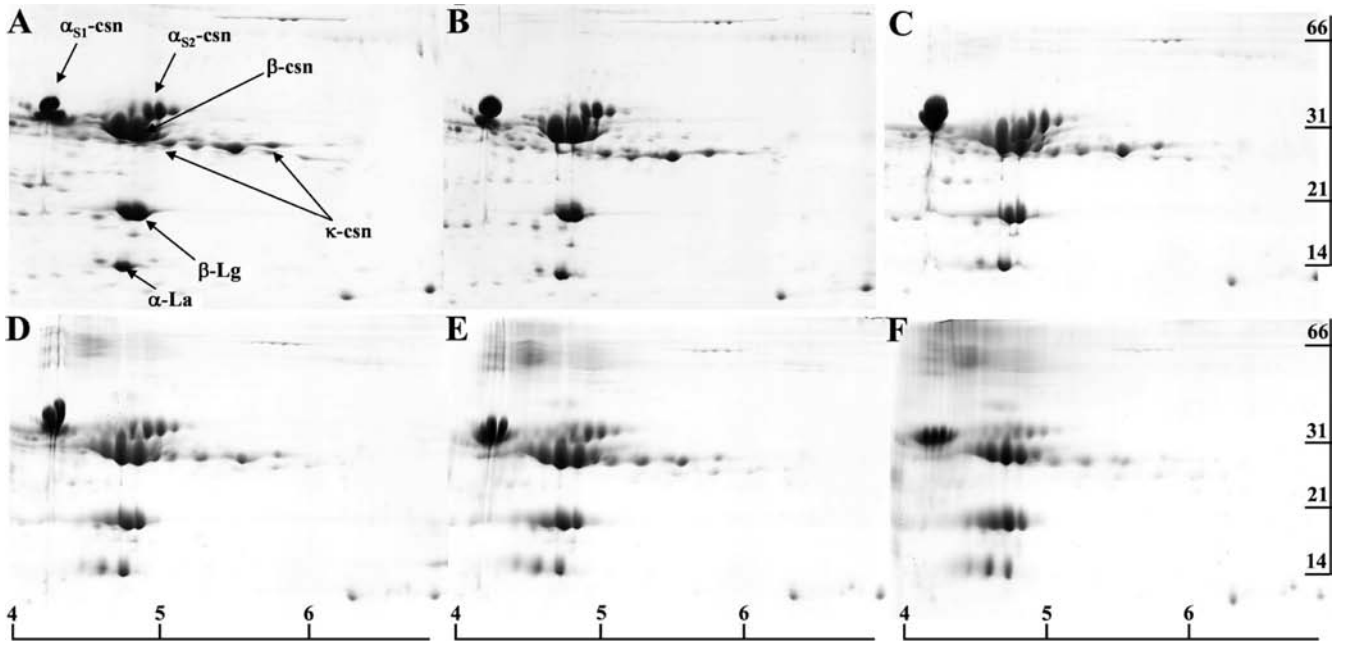

Figure 1. 2-D gels of milk before and after UHT treatment and storage. Samples were focused using pH 4−7 strips and electrophoresed on 14% polyacrylamide gels as described: (A) unprocessed whole milk; (B) UHT treated milk; (C−F) UHT treated milk stored for two months at (C) 4 °C, (D) 28 °C, (E) 28 °C after initial storage for two weeks at 40 °C and (F) 40 °C. The vertical scale shows apparent molecular mass in kDa, and the horizontal scale shows pH.

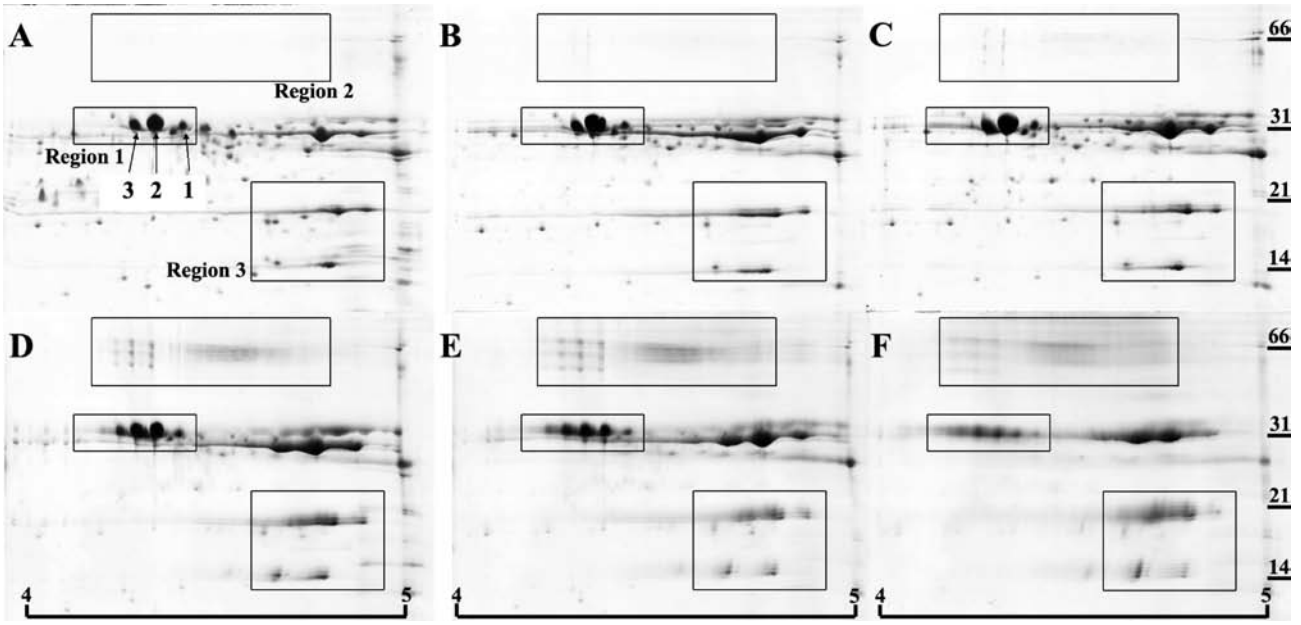

Figure 2. 2-D gels showing the protein separation on a narrow range pH 4−5 strip with extended focusing hours. Samples were focused using pH 4−5 IPG strips and electrophoresed on 14% polyacrylamide gels as described: (A) unprocessed whole milk; (B) UHT treated milk; (C−F) UHT treated milk stored for two months at (C) 4 °C, (D) 28 °C, (E) 28 °C after initial storage for two weeks at 40 °C and (F) 40 °C. Selected regions are designated by the boxes and labeled 1, 2, and 3 in (A). The vertical scale shows apparent molecular mass in kDa, and the horizontal scale shows pH.

of non-disulfide cross-linking, deamidation and lactosylation of milk proteins during storage.

MATERIALS AND METHODS

Materials. Milk samples were obtained from the University of Queensland's Gatton dairy herd and processed as described below. Immobiline DryStrips (pH 4−7 and pH 4−5, 24 cm) and IPG buffer (pH 4−7 and pH 3.5−5.0) were obtained from GE Healthcare (Sydney, Australia). Modified porcine trypsin (proteomics grade) was obtained from Sigma (Sydney, Australia). α-Cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were obtained from Sigma (Sydney, Australia). All other reagents were of analytical grade or better.

Table 1. Peptide Mass Fingerprinting of α_{S1}-Casein[a]

mass[b] (Da)			
exptl	theor	residues[c]	peptide sequence[d]
2235.2302	2235.2356	19−37	HPIKHQGLPQEVLNENLLR
1759.9424	1759.9449	23−37	HQGLPQEVLNENLLR
1384.7284	1384.7299	38−49	FFVAPFPEVFGK
946.5235	946.5204	50−57	EKVNELSK
2598.0713	2598.0565	52−73	VNELSKDIGsEsTEDQAMEDIK
2614.0591	2614.0514	52−73	VNELSKDIGsEsTEDQAmEDIK
1927.6908	1927.6915	58−73	DIGsEsTEDQAMEDIK
1943.6923	1943.6864	58−73	DIGsEsTEDQAmEDIK
2720.9290	2720.9129	74−94	QMEAEsIsssEEIVPNsVEQK
1337.6794	1337.6807	95−105	HIQKEDVPSER
831.3940	831.3842	99−105	EDVPSER
1267.7046	1267.7044	106−115	YLGYLEQLLR
2080.0498	2080.0709	118−134	KYKVPQLEIVPNsAEER
1951.9481	1951.9524	119−134	YKVPQLEIVPNsAEER
1660.7916	1660.7941	121−134	VPQLEIVPNsAEER
910.4839	910.4740	140−147	EGIHAQQK
3207.5950	3207.5931	140−166	EGIHAQQKEPMIGVNQELAYFYPELFR
3223.6055	3223.5880	140−166	EGIHAQQKEPmIGVNQELAYFYPELFR
2316.1367	2316.1368	148−166	EPMIGVNQELAYFYPELFR
2332.1321	2332.1317	148−166	EPmIGVNQELAYFYPELFR
4716.2534	4716.1728	167−208	QFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEK
748.3676	748.3698	209−214	TTMPLW
764.3605	764.3647	209−214	TTmPLW

[a] Tryptic digests were analyzed in reflector mode using 2,5-dihydroxybenzoic acid as the matrix. [b] Monoisotopic mass, $[M + H]^+$. Experimental values are from spot 2. [c] Numbering from UniProtKB entry P02662 includes the signal peptide, residues 1−15. [d] Modified residues are shown with lowercase letters: s, phosphoserine; m, methionine sulfoxide.

UHT Treatment. Milk samples were preheated at 100 °C for 30 s and UHT treated at 136 °C for 4 s in an APV 100 L/h tubular UHT pilot plant. The milk was packaged aseptically into 2 L Intasept bags (VisyPak, Melbourne, VIC, Australia). Unprocessed milk and milk samples immediately after the UHT treatment (control) were stored at −80 °C. UHT treated samples were stored at three different temperatures, 4 °C, 28 °C and 40 °C, for two months. Milk stored at these temperatures are reported to have low, high and low susceptibilities, respectively, to gelation.[3] An additional set of samples was returned to 28 °C after two weeks of storage at 40 °C. This set was included because it has been suggested that storage at ∼40 °C may cause changes in the micelle, such as cross-linking, which could prevent gelation.[40]

After the designated storage time, samples were aliquoted and stored at −80 °C. All samples after UHT treatment, with and without storage, were liquid with no obvious color differences. No gelation was observed after 2 months' storage.

2-DE. Samples of milk (30 μL) were mixed with solubilization solution (8 M urea, 4% CHAPS, 0.5% appropriate IPG buffer and 100 mM DTT) to a final volume of 450 μL. The solubilized samples were used to hydrate IPG strips at room temperature for a minimum of 8 h. Isoelectric focusing (IEF) of strips was performed on IPGphor (Amersham Biosciences, Sydney Australia) at 100 V for 1 h, followed by 500 V for 1 h and 1 kV for 1 h, before increasing the voltage to 8 kV, for a total of 100 kV·h for pH 4−7 strips or 150 kV·h for pH 4−5 strips. Focused strips were stored at −20 °C before running the second dimension. For the second dimension, the strips were equilibrated for 10 min in equilibration buffer (6 M urea, 2% SDS, 20% glycerol and 0.375 M Tris-HCl, pH 8.8) containing 15 mg/mL DTT, followed by 20 min in the equilibration buffer containing 20 mg/mL iodoacetamide. Equilibrated strips were embedded onto 25 × 20 cm 14% polyacrylamide gels using 0.5% agarose and electrophoresed at 5 mA/gel for 2 h followed by 20 mA/gel for 14 h. Gels were stained with colloidal Coomassie blue G-250 for 24 h and destained with 1% acetic acid. Images were obtained on an ImageScanner (Amersham Biosciences) in transmission mode at a resolution of 300 dpi. Milk samples from 2 separate UHT trials were analyzed in triplicate. Representative gels are shown in the figures.

In-Gel Tryptic Digestion. In-gel digests were performed as described previously using trypsin.[31] Briefly, selected gel plugs were washed with water and destained using two washes (100 μL) of 40 mM ammonium bicarbonate in 50% acetonitrile (ACN) for 30 min each. Plugs were dehydrated with 200 μL of 100% ACN for 10 min and rehydrated with 20 μL of trypsin solution (10 μg/mL in 40 mM ammonium bicarbonate, pH 8) and incubated overnight at 37 °C. Peptides were extracted twice with 20 μL of 5% formic acid/50% ACN, dried in a vacuum centrifuge and stored at −20 °C if not analyzed immediately by mass spectrometry.

Mass Spectrometry. Peptide digests were redissolved with 1% formic acid in 50% ACN and mixed with an equal volume of matrix solution, and 1 μL was spotted on a stainless steel MALDI target. Spectra were acquired using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) in delayed extraction mode. Tryptic digests were analyzed in positive ion reflector mode with an accelerating voltage of 20 kV, grid voltage at 64% and a delay time of 165 ns. Four hundred laser shots were accumulated for each spectrum. The matrix was either 10 mg/mL CHCA in 1% formic/50% ACN or 20 mg/mL DHB in 5 mM NH_4HPO_4/1% phosphoric acid/50% ACN. Spectra were internally calibrated with trypsin autolysis peaks or known

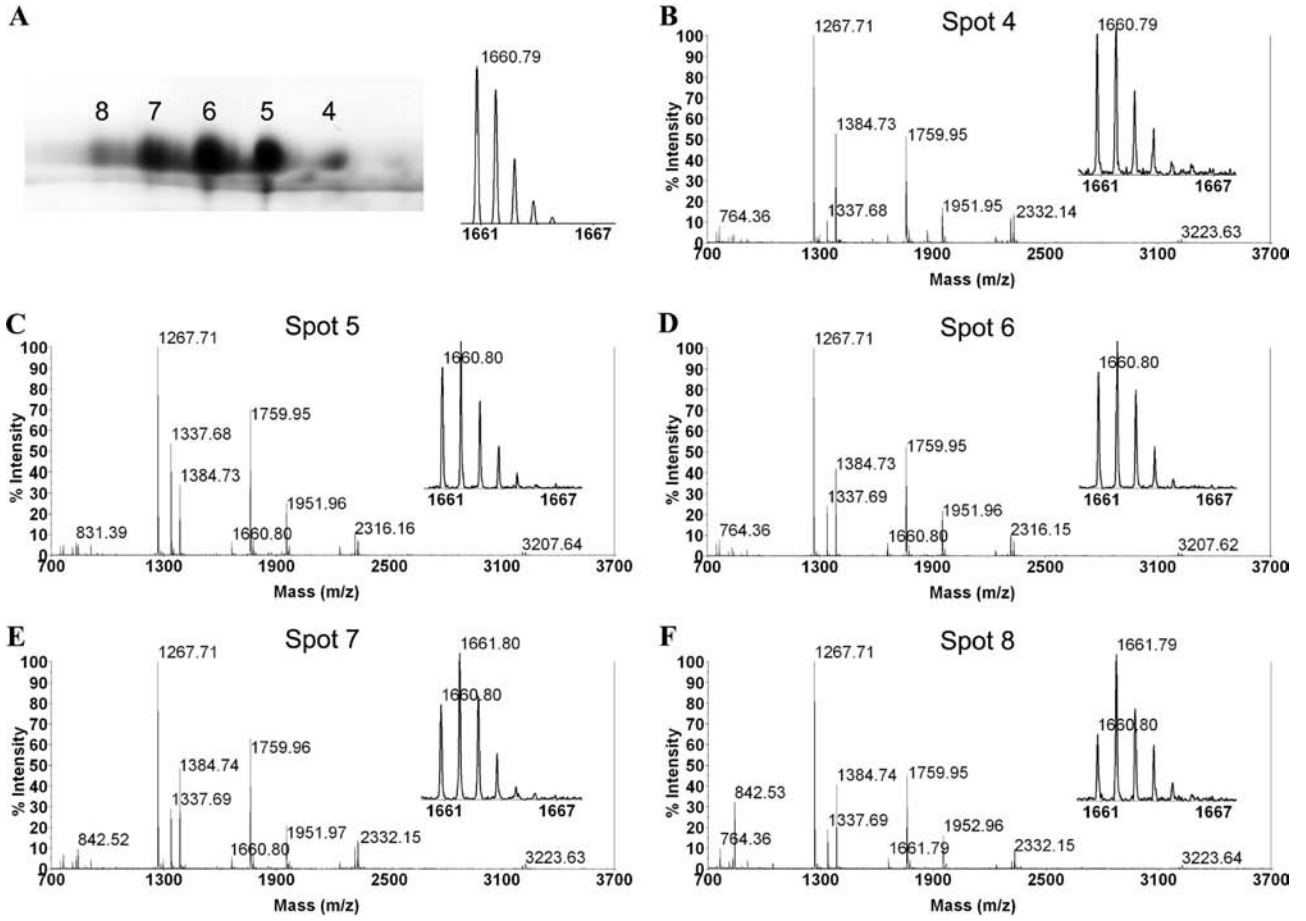

Figure 3. Deamidation of α_{S1}-csn in stored UHT milk. (A) Enlarged view of region 1 from Figure 2E. (B−F) Mass spectra of tryptic digests from spots 4−8 in panel A. The inset in panel A shows the theoretical isotopic profile of the peak at m/z 1660.8 from the peptide ^{121}VPQLEIVPNsAEER^{134}. Insets in panels B−F show the profile observed for spots 4−8.

peaks from the proteins of interest. MS/MS of peptides was performed on ABI 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) as described previously.[41] Initial MS spectra were acquired manually in reflector positive operating mode with source voltage 20 kV and grid 1 voltage 12 kV, mass range 500−5000 Da, focus mass 1500 Da, collecting 1000 shots. MS/MS spectra were acquired manually with source voltage 8 kV and grid 1 voltage 6.8 kV, precursor mass window set to relative 200 fwhm, collecting a minimum of 2000 shots.

RESULTS AND DISCUSSION

Analysis of UHT Treated Milk by 2-DE. The effects of UHT treatment and storage conditions on the milk proteins were investigated by 2-D electrophoresis. Milk samples after UHT treatment were compared with unprocessed whole milk. Figure 1 shows gels from unprocessed milk (Figure 1A) and milk immediately after UHT treatment (Figure 1B) focused on pH 4−7 IPG strips. The prominent spots on the gels are from the major milk proteins α_{S1}-casein (α_{S1}-csn), α_{S2}-casein (α_{S2}-csn), β-casein (β-csn) κ-casein (κ-csn), α-lactalbumin (α-La) and β-Lg. The gel from a freshly UHT treated sample (Figure 1B) was similar when compared with the gel from unprocessed milk (Figure 1A). The above samples also showed no major differences when examined over a broader pH range (IPG strips pH 3−10, data not shown). The above results suggest that any initial effects of the UHT treatment did not alter the electrophoretic mobility of the milk proteins.

Gels from UHT milk stored for two months at 4 °C, 28 °C, 40/28 °C or 40 °C are shown in Figures 1C−1F respectively. Although analyzed under identical conditions, resolution of the proteins differed between samples, with the proteins being better resolved in gels from the sample stored at 4 °C (Figure 1C) than 28 °C and 40 °C (Figures 1D−1F). This suggests that after two months, in different storage conditions, milk proteins undergo physicochemical changes and modifications leading to changes in their electrophoretic behavior. Apart from the resolution, other differences were observed and were more prominent in the gels from samples stored at elevated temperatures, 28 °C and 40 °C. The gels from samples stored at 40 °C showed extra spots appearing toward the more acidic end of the gel. This can be clearly seen in whey proteins and α_{S1}-csn.

To obtain better resolution of the protein spots, especially for the samples stored at elevated temperatures, the samples were focused using narrow range IPG strips (pH 4−5) for the first dimension. The gels (Figure 2) showed significant improvement in both resolution and separation of the multiple protein spots compared to the pH 4−7 gels in Figure 1. Three regions showing major differences were defined as indicated in Figure 2 (similar to the approach used by Chevalier et al.[39]) and will be discussed below.

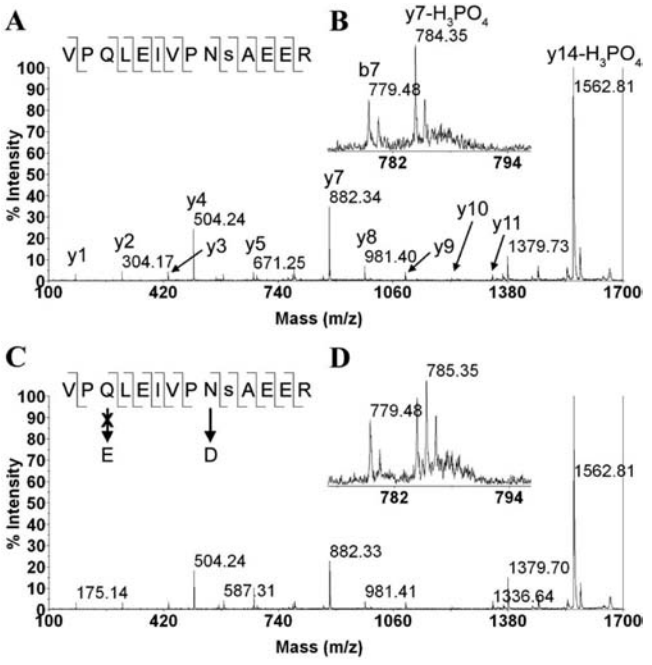

Figure 4. Deamidation of N^{129} in α_{S1}-csn. (A,C) MS/MS spectra of ion at m/z 1660.8 from the peptide ^{121}VPQLEIVPNsAEER^{134} in digests from spots 4 (A) and 8 (C) in Figure 3. (B, D) Inset showing b7 and y7-H_3PO_4 ions from spots 4 and 8, respectively. The fragment ions are labeled in (A). Note the change in isotopic profile of y7- H_3PO_4 at m/z 784.35 but not in b7 at m/z 779.48 indicating partial deamidation at N^{129} but not Q^{123}.

Protein Changes in Region 1. Region 1 contains one of the most abundant milk proteins, α_{S1}-csn. The major form of α_{S1}-csn is the B variant with 8 phosphates attached (α_{S1}-csn B-8P) but there is also a minor component with 9 phosphates.[42] Three spots, labeled 1−3 in Figure 2A, were subjected to in-gel digestion with trypsin, and the peptides released were analyzed by MALDI-TOF MS. The identified peptides are summarized in Table 1. Only the short fragments, R^{16}−K^{18}, L^{116}−K^{117} and L^{135}−K^{139}, were not observed giving sequence coverage of 95%. However, the multiply phosphorylated peptides, D^{58}−K^{73} and Q^{74}−K^{94}, had very low intensities and were not consistently observed in the weaker α_{S1}-csn spots. Spot 2 was identified as the major (8P) form. Spot 3 was consistent with the minor (9P) form, and spot 1, which had a less acidic p*I*, was consistent with the 7P form.

The pattern of α_{S1}-csn spots was similar in milk immediately after UHT treatment (Figure 2B) and after storage at 4 °C (Figure 2C), but changed in gels from samples stored at elevated temperatures (Figures 2D−2F). The major α_{S1}-csn spot (8P) showed a gradual decrease in size with increasing storage temperature, and there was a corresponding increase in the size of the minor spot (9P) beside it (Figures 2D−2F). Additionally, more acidic spots were observed in the gels from samples stored for two months at elevated temperatures (Figures 2D−2F).

To examine the changes further the 5 main spots in region 1 from Figure 2E were subjected to in-gel digestion with trypsin and the peptides released were examined by MS (Figure 3). The spectra showed similar MALDI-TOF mass spectra. The same major ions were consistently seen for all 5 spots. Closer examination of the spectra revealed differences in the isotopic profiles of some ions. Figures 3B−3F shows the ion at m/z of 1660.8 from the peptide, ^{121}VPQLEIVPNsAEER^{134}, for spots 4−8 respectively. A progressive decrease in the size of the monoisotopic peak relative to the ^{13}C peak can be seen as the spots become more acidic. The pattern is indicative of deamidation when partial conversion of an asparagine residue to an aspartate (or glutamine to glutamate) produces a peptide ion with an isotopic cluster shifted 1 Da higher than the nondeamidated peptide. As the two isotopic clusters overlap, the shape of the profile is determined by the relative amounts of the native and deamidated peptides.

Nonenzymatic deamidation of asparagine or glutamine occurs following reaction between the side chain amide and the succeeding backbone amide producing a cyclic imide that hydrolyzes to produce the corresponding acid. The highest rates are observed for asparagine residues that are followed by glycine, but significant levels are seen with serine or histidine in place of glycine[43−45]. It appears that phosphoserine is also favorable in the +1 position.

The ion at m/z 1660.8 from the peptide ^{121}VPQLEIVPNsAEER^{134} was examined by MS/MS to confirm the site of deamidation. Figure 4A shows the MS/MS spectra obtained for the peptide from spot 4 in Figure 3. The pattern of b- and y-ions confirms the identity of the peptide. The inset (Figure 4B) is an expansion to show the b7 and y7-H_3PO_4 ions that contain Q^{123} and N^{129} respectively. Figure 4C shows the MS/MS spectrum from spot 8, and the inset (Figure 4D) clearly shows the change in isotopic profile of the y7-H_3PO_4 ion but not the b7 ion, confirming N^{129} as the site of deamidation.

The deamidation of N^{129} shown in Figure 3 was only partial even in the most acidic spot and therefore insufficient to account for all the additional acidic forms of α_{S1}-csn. There are 2 other asparagine residues in α_{S1}-csn that are followed by (phospho-) serine and therefore likely candidates for additional deamidation. They could not be confirmed as N^{89}−S^{90} is in the multiphosphorylated peptide, Q^{74}−K^{94}, which was not consistently observed and N^{205}−S^{206} occurs in the large peptide, Q^{167}−K^{208}, which was not always resolved sufficiently to observe a change in the isotopic profile.

Protein Changes in Region 2. Region 2 shows apparent higher molecular weight protein complexes, particularly in gels from samples stored at elevated temperatures (Figures 2D−2F). There were 2 major components in this region: first, a group of partially resolved spots located immediately above the main α_{S1}-csn spots and, second, an area of diffuse staining with a p*I* range intermediate between α_{S1}-csn and β-csn. Both components were subjected to in-gel digestion, and the peptides released were examined by MS to determine the proteins present (Figure 5). The spots above α_{S1}-csn were also identified as α_{S1}-csn and appeared to be oligomeric forms of the protein based on their electrophoretic mobility. The spectra showed no apparent differences between the monomer (spot 9, Figure 5B) and oligomers (e.g., spot 10, Figure 5C). The spectrum obtained from the diffusely stained area (spot 13) contained peaks corresponding to a mixture of peptides derived from α_{S1}-csn, α_{S2}-csn (see spot 11) and β-csn (see spot 12) (Figures 5D−5F). This region appears to be a mixture of complexes between α_{S1}-csn, α_{S2}-csn and/or β-csn that is consistent with the horizontal position on the gel between the α_{S1}-csn and α_{S2}-csn (or β-csn) monomers. The gels were run under denaturing conditions following reduction and alkylation of cysteines so the complexes are covalently linked but not via disulfide bonds. The resolution in this region was poor, suggesting that a fairly heterogeneous population of protein species was present.

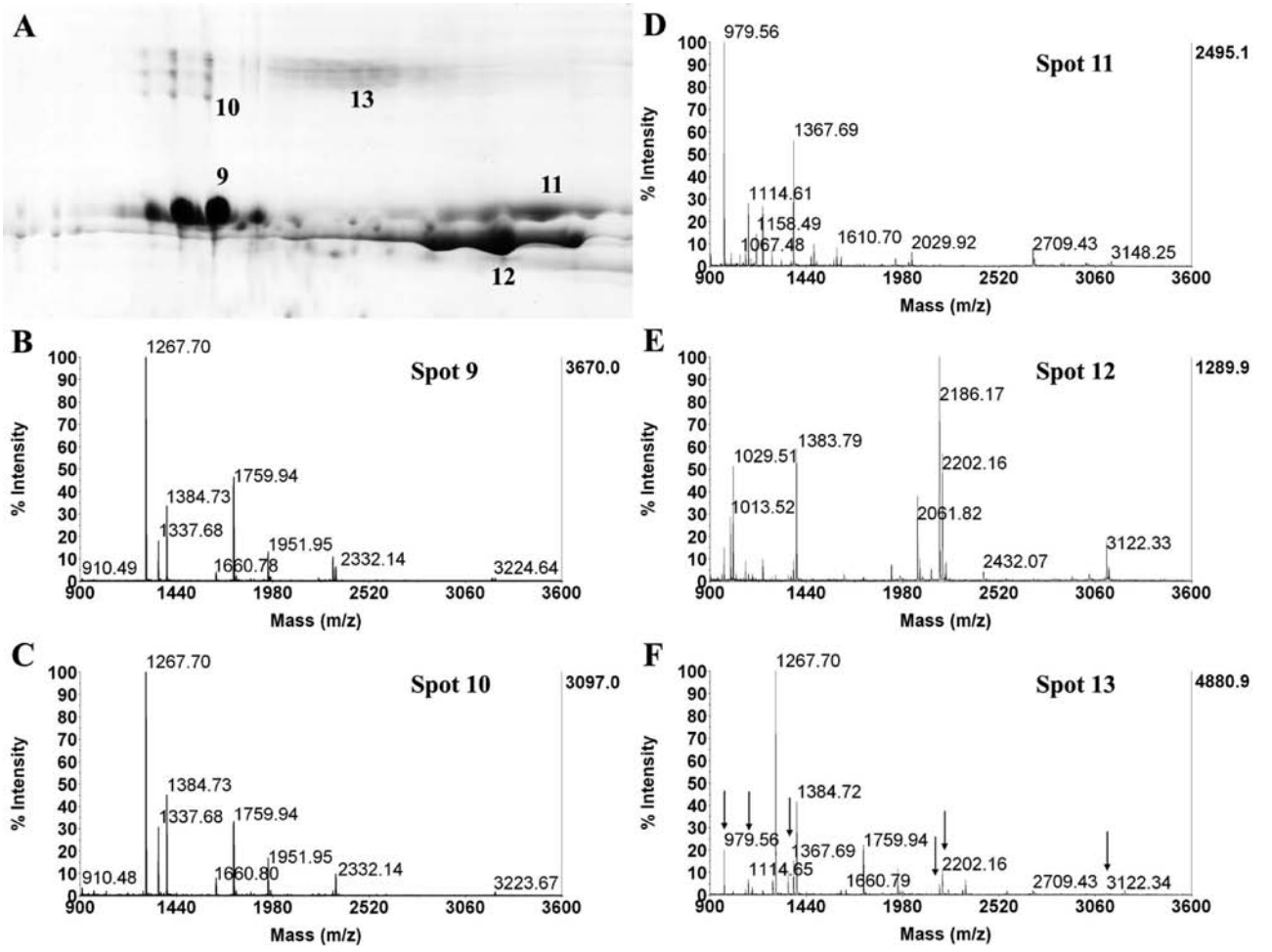

Figure 5. Protein cross-linking. (A) 2D gel of UHT treated milk after storage at 28 °C for 2 months. The gel was run under reducing conditions to eliminate disulfide-linked complexes. (B−F) MS of tryptic digests of spots indicated in panel A. Arrows indicate peaks from α_{S2}-csn and β-csn in panel F.

The most likely explanation for these complexes is the formation of lysinoalanine cross-links between the proteins.[46] Heating of casein can cause the loss of phosphate from phosphoserine residues via a β-elimination mechanism to form dehydroalanine. Subsequent reaction with the ε-amino group of a lysine residue produces lysinoalanine cross-links. Cross-links via histidine or cysteine are also possible, but the higher abundance of lysine and the absence of cysteine in α_{S1}-csn and β-csn make lysinoalanine the most likely candidate. As a number of different phosphoserine and lysine residues could be involved, a very heterogeneous population of cross-linked forms could result. Subtle differences in the electrophoretic mobility and/or effective p*I* of the oligomers would contribute to the poor resolution seen on the gels.

No peaks in the spectra of the digests of the oligomers could be assigned to cross-linked peptides so the presence of lysinoalanine could not be confirmed. However, cross-linked peptides are not always easy to observe by MS.[47] In addition, phosphorylated peptides, especially the multiply phosphorylated ones typical of caseins, ionize poorly and are also difficult to detect by MS.[41,48] Consequently, detection of cross-linked, phosphorylated peptides is likely to be very difficult.

An alternative mechanism for the cross-linking observed on 2D gels could be via Maillard reaction products, as proposed recently.[39,49] A number of cross-linking mechanisms are possible, but mostly they involve advanced or late Maillard products.[4] As the level of lactosylation observed here was still quite low, the participation of advanced Maillard products seems less likely. However, a definitive answer is not possible at this stage, and multiple mechanisms could have been operating.

Protein Changes in Region 3. The use of narrow range IPG strips (Figure 2) also revealed more detail of the changes occurring in the major whey proteins, α-La and β-Lg in region 3 on the gels. Two effects of increasing storage temperature were apparent on the gels. First, there appeared to be additional spots to the left similar to the deamidation effects seen for α_{S1}-csn in region 1. Second, the individual spots seen in region 3 of Figures 2A−2C (unprocessed milk, milk immediately after UHT treatment and milk stored at 4 °C) appeared as vertical stacks of spots in the UHT treated milk stored at higher temperatures (Figures 2D−2F). These effects can be seen in more detail in Figure 6 which shows region 3 on the gels from Figure 2A (unprocessed milk) and Figure 2E (UHT milk stored at 40 °C/28 °C for 2 months. The arrows in Figure 6A show that the major whey proteins (β-Lg A, β-Lg B and α-La) were resolved as single spots. In Figure 6B, they are replaced by 5 stacks of spots for β-Lg (labeled 14−18) and 2 stacks for α-La (labeled 19−20). The B variant of β-Lg contains 5 asparagines but only 2 in likely nonenzymatic deamidation motifs, N^{79}−G^{80} and N^{125}−S^{126}. The A variant of β-Lg has aspartate in place of

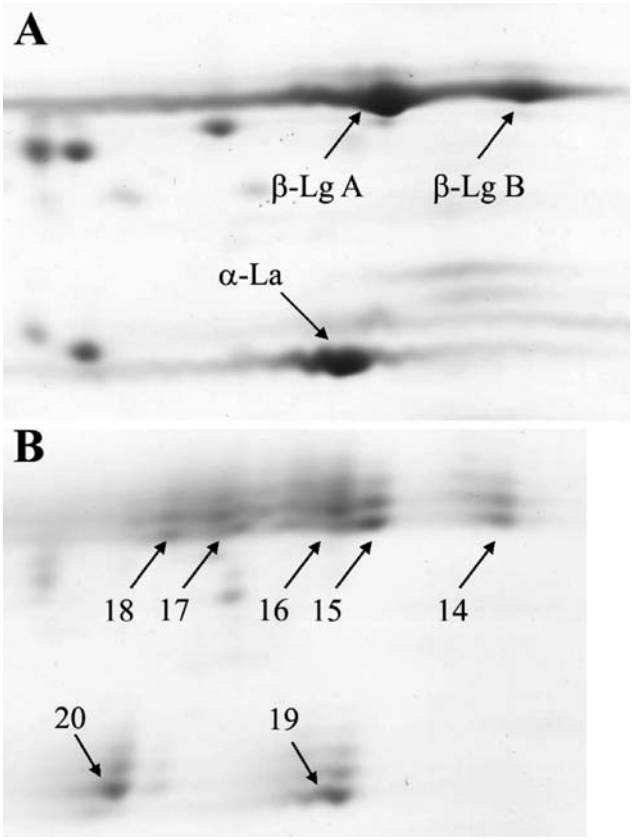

Figure 6. Changes in major whey proteins. (A, B) Enlarged views of region 3 from the gels shown in Figure 2A (unprocessed whole milk) and 2E (2 months at 40 °C/28 °C) respectively. The arrows in panel A show good separation of the major whey proteins, β-Lg A, β-Lg B and α-La in unprocessed whole milk. A complex picture of modifications in whey proteins was obtained in the samples stored for two months at 28 °C after initial storage for two weeks at 40 °C after UHT treatment; arrows 14−18 show spots identified as β-Lg, and arrows 19 and 20 show spots identified as α-La.

glycine at position 80 so it contains only 1 likely deamidation site. The combination of deamidated and nondeamidated forms could explain the number of stacks observed in Figure 6B. Deamidation of α-La is harder to explain. Although it contains 8 Asn residues, none of them occur in recognized deamidation motifs.

The multiple spots observed in Figure 6B were rather weak, and although sufficient peaks were observed after in-gel digestion to confirm them as β-Lg or α-La, the spectra were too weak to identify differences between the individual isoforms. The samples were re-examined on 2-D gels using pH 4−7 IPG strips. Figure 7A shows a small section of a gel similar to that in Figure 1E. Although some resolution was lost relative to the gel using pH 4−5 IPG strips, the spots were more intense. Spots 21−23 in Figure 7A were identified as β-Lg, and their spectra are shown in Figures 7B−7D, respectively. The spectrum of spot 21 contained a peak at m/z 1121.47 (^{77}WENGECAQK^{85}) from β-Lg B, and its theoretical isotopic profile is shown in the inset to Figure 7A. Spots 22 and 23 also contained the β-Lg B peak and an additional peak at m/z 1179.48 (^{77}WENDECAQK^{85}) from β-Lg A. Examination of the isotopic profile of the β-Lg B peak in the insets to Figures 7B−7D showed an increasing level of deamidation as the spots became more acidic. Interestingly, partial

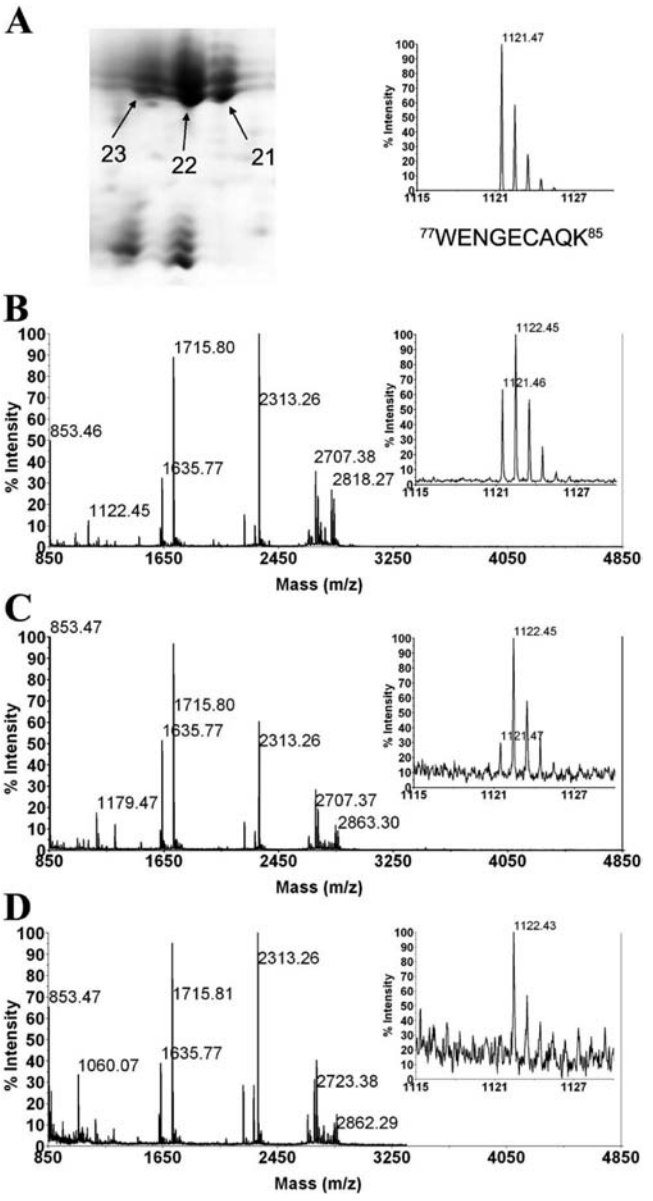

Figure 7. Deamidation of β-Lg in stored UHT milk. (A) Small section of 2D gel similar to Figure 1E. (B−D) MS of digests of spots 21−23 respectively. Insets show the isotopic profile for the peptide ^{77}WENGECAQK^{85} at m/z 1121.47: (A) theoretical isotopic profile; (B−D) observed profiles for spots 21−23, respectively.

deamidation was observed even in spot 21, where no shift had occurred. Nonenzymatic deamidation of asparagine at NX motifs occurs readily in peptides where X is glycine, with a half-life of the order of 1 day at 37 °C.[50] So deamidation at N^{79} in spot 21 probably occurred during overnight tryptic digestion. Where X is not glycine, the half-life is on the order of 10 days or more, so significant levels of deamidation at other asparagine residues during digestion would have been unlikely. Deamidation at N^{79} was confirmed by MS/MS of the 1121.47 peak from the digest of spot 21. Figure 8A shows a full y-ion series for the peptide ^{77}WENGECAQK^{85}. Figure 8B shows a normal isotopic profile for the y6 ion whereas Figure 8C shows clear evidence of deamidation for y7, confirming the deamidation site as N^{79}. The presence of the β-Lg B peak in all 3 spots cannot be explained entirely by deamidation of N^{79}. A second deamidation

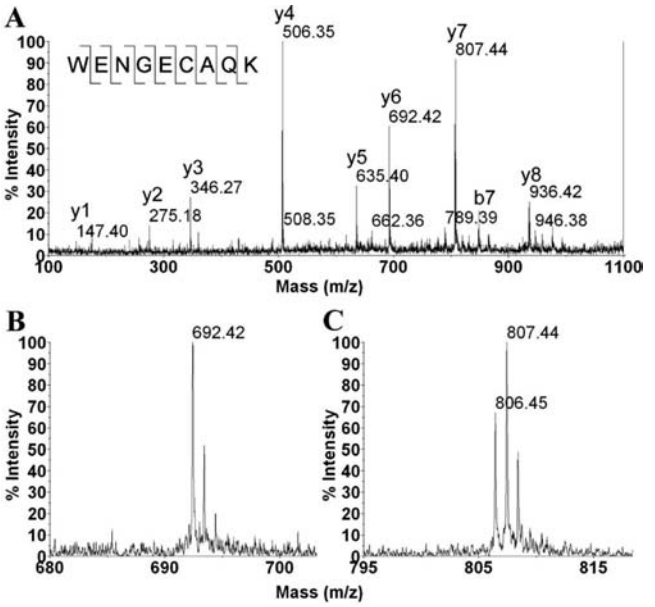

Figure 8. Deamidation of N^{79} in β-Lg. (A) MS/MS of β-Lg peak at m/z 1121.47 corresponding to the peptide ^{77}WENGECAQK^{85} in the digest of spot 21. (B) Expansion showing the isotopic profile of y6 ion with no evidence of deamidation. (C) Expansion showing the isotopic profile of y7 ion with clear evidence of deamidation. Partial deamidation of y7 but not y6 confirms that deamidation occurred at N^{79} but not Q^{84}.

site would be required. Inspection of the spectra did not reveal any other obvious deamidation, so other chemical changes could be occurring.

The second effect in region 3 of the 2-D gels was of vertical "spot stacking". One explanation for this effect is lactosylation of the proteins, an effect which has been widely reported in heat-treated milk and milk products, particularly for whey proteins. Lactosylation would increase the mass of the protein by 324 Da. For relatively small proteins like β-Lg (18 kDa) and α-La (14 kDa) the mass difference would be sufficient for the differentially lactosylated proteins to be resolved on the large format (25 × 20 cm) gels. Each lactose molecule would add an extra spot in the stack.

To confirm lactosylation, digests of β-Lg and α-La spots from the bottom of the stacks (Figure 9A, spots 25 and 27) were compared with the spots above them (Figure 9A, spots 24 and 26). A number of additional peptides were observed in the upper spots for both β-Lg (Figures 9 B and 9C) and α-La (Figures 9D−9E). These peptides all corresponded to predicted peptides in the lower spots but had mass increases of 324 Da indicating lactosylation had indeed occurred.

Lactosylation is the first step of the Maillard reaction in milk and occurs on lysine residues. Although the gel shifts from spot 25 to 24 and spot 27 to 26 were consistent with a single lactose adduct on the protein, the peptides in Figure 9 show lactosylation on K^{24}, K^{99} and K^{151} within β-Lg and K^{117}, K^{127} and K^{133} within α-La. Overall there was evidence for lactosylation on 9 of the 15 lysine residues in β-Lg and 8 of the 12 lysines in α-La (see Supporting Information). It appears that most if not all of the lysine residues in β-Lg and α-La were susceptible to lactosylation that would fit with the surface location of these residues in the 3-D structures in the Protein Data Bank and with previous reports of heterogeneous lactosylation sites in whey proteins.[7,19,22] Lactosylation was only observed on peptides

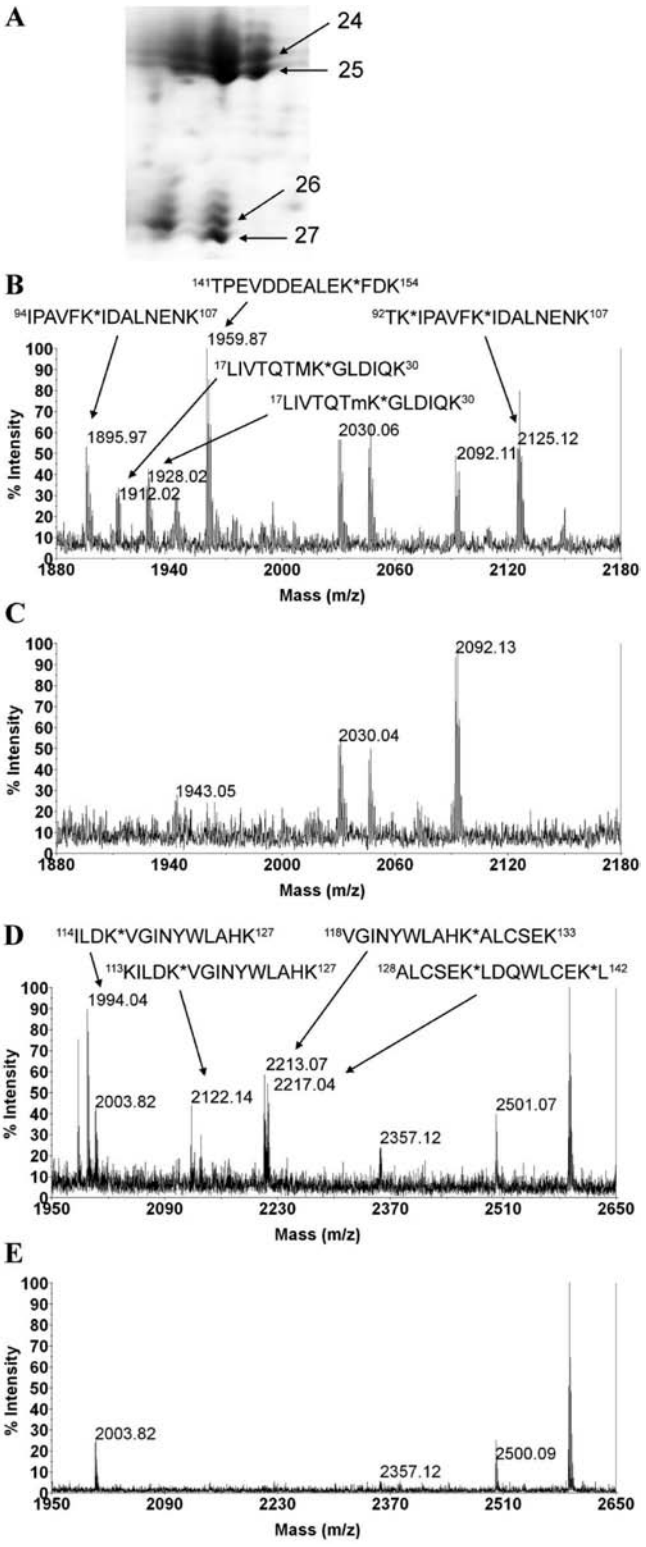

Figure 9. Lactose adducts on whey proteins. (A) Small section of 2D gel similar to Figure 1E. (B−E) MS of digests of spots 24−27 respectively. Peaks corresponding to lactose adducts on peptides from β-Lg (spot 24) and α-La (spot 26) are labeled in panels B and D, respectively.

containing a missed cleavage at an internal lysine residue so we can conclude that lactosylation prevents cleavage by trypsin at

the modified residues as reported earlier.[22] So apart from the decrease in nutritional value caused by the modification of the lysine residues, a decrease in digestibility is also likely to be a consequence of the extensive lactosylation seen in the whey proteins of UHT milk stored at elevated temperatures.

In conclusion, the results shown here demonstrate that 2-DE is a powerful technique to visualize the changes that occur in UHT-treated milk during storage at elevated temperatures. When coupled with MS, specific molecular changes can be identified from changes in m/z and tandem MS can be used to assign changes to specific sites on the proteins. The technique resulted in the identification of three major changes that occur simultaneously in UHT milk during storage, deamidation, non-disulfide cross-linking and lactosylation. Interestingly, there was no evidence for these changes in freshly prepared UHT milk. They only became manifest upon storage, and substantial levels were only observed in UHT milk stored at elevated temperatures. This finding is particularly relevant with regard to lactosylation. A number of recent studies have reported substantial levels of lactose adducts on whey proteins in UHT milk.[7,13,18] Our results suggest that aging rather than processing could be the biggest contributor. The idea of measuring Maillard reaction products as markers of milk thermal history has been around for a long time, but it is important to remember that the thermal history of milk and milk products also includes storage time and temperature as noted earlier.[4] Consequently, any changes to processing conditions designed to minimize the Maillard reaction may have little effect on product quality if subsequent storage conditions are less than ideal.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Tables listing lactosylated peptides identified by Mascot. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

2-DE, two-dimensional electrophoresis; UHT, ultrahigh temperature; β-Lg, β-lactoglobulin; α-La, α-lactalbumin; ACN, acetonitrile; CHCA, α-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid.

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