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ARTICLE

Use of Reducing/Nonreducing Two-Dimensional Electrophoresis for the Study of Disulfide-Mediated Interactions between Proteins in Raw and Heated Bovine Milk

François Chevalier,*,† Christophe Hirtz,§ Nicolas Sommerer,§ and Alan L. Kelly†

†Department of Food and Nutritional Sciences, University College Cork, Cork, Ireland, and §Proteomic Platform, INRA, Montpellier, France

The composition and interactions of proteins in bovine milk, and modifications resulting from milk storage and processing, are complex and incompletely understood. Analysis of the milk proteome can elucidate milk protein expression, structure, interaction, and modifications. Raw milk was analyzed by two-dimensional electrophoresis (isolelectric focusing followed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis) under reducing and nonreducing, or combined, conditions, followed by mass spectrometry of separated protein spots; a small number of high-abundance proteins, that is, caseins (α_{S1}-, α_{S2}-, β-, κ-, and γ-), β-lactoglobulin, α-lactalbumin, and serum albumin, represented the vast majority of protein spots on the two-dimensional electrophoretograms of raw milk samples, but some cross-linked protein complexes (mainly homopolymers of κ-casein and α_{S2}-casein but also some heteropolymeric complexes) were resolved under native/unheated conditions. When skim milk was heated to 90 °C for up to 10 min, the level of native whey proteins decreased in parallel with an increase in disulfide-linked complexes, including very complex heteropolymers, for example, casein/whey protein polymers containing multiple species. The analysis strategy used in this study reveals numerous disulfide-mediated interactions and can be proposed to analyze reduction/oxidation of milk and dairy product proteins following processing treatments applied for processing and storage.

KEYWORDS: Milk proteins; proteome; interactome; disulfide bridges; heat treatment; mass spectrometry

INTRODUCTION

Heat treatment is applied in the processing of milk for both hygienic and technological reasons and is today an essential operation in commercial dairy processes to provide acceptable safety and shelf life of dairy products. As a consequence of heat treatment, milk proteins may undergo structural changes, such as unfolding and aggregation. Heat treatment of milk at >70 °C results in a number of physicochemical changes in the milk constituents, in particular, denaturation of whey proteins and the formation of hydrophobic interactions or disulfide-bonded aggregates with casein micelles, particularly through interactions with κ-casein. Such protein−protein interactions can significantly affect the stability of milk and contribute to heat-induced coagulation of the milk. Numerous studies have been performed on model systems to elucidate the mechanism of interactions between milk proteins after heat treatment (*1*−*5*).

Zittle et al. (*6*) provided the first conclusive evidence of complex formation between β-lactoglobulin (β-LG) and κ-casein (κ-CN). Sawyer et al. (*7*) demonstrated the involvement of thiol groups and suggested that the free thiol group of β-LG is involved in the interaction and that intermolecular disulfide bonds are formed between κ-CN and β-LG. Subsequent investigations have tended to focus on determining the types of bonding involved in complex formation and the stoichiometry of the complexes formed (*5*, *8*−*16*). Sawyer (*7*) and McKenzie et al. (*17*) showed that the self-aggregation of β-LG was limited when κ-CN was present and suggested that κ-CN forms complexes with intermediate species of aggregated β-LG. In contrast, Euber and Brunner (*14*) reported that the aggregation of β-LG is not a prerequisite for interaction with κ-CN. Cho et al. (*5*) examined the interaction of β-LG with κ-CN using a "natural" κ-CN isolated without reduction or chromatography. Only denatured β-LG interacted with κ-CN (*18*), and many of the possible pathways involved in the aggregation of β-LG with κ-CN were elucidated. It has been proposed that, on heating β-LG and κ-CN, the free thiol group of β-LG is exposed, which initiates a series of reactions with other denatured molecules of β-LG or with κ-CN. The products formed depend on the ratio of κ-CN to β-LG and included 1:1 β-LG−κ-CN complexes and a range of large heterogeneous aggregates that were held together by disulfide bonds and/or hydrophobic interactions (*7*, *19*−*21*).

*Author to whom correspondence should be addressed [telephone +33 (0)146 548 326; fax +33 (0)146 549 138; e-mail francois.chevalier@cea.fr].

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Milk is considerably more complex than purified protein model systems. Although β-LG is the major whey protein in bovine milk, several other whey proteins with free thiol groups and/or disulfide bonds are present, such as serum albumin and α-lactalbumin (*22*). Of the caseins, both κ-CN and α_{S2}-CN have disulfide bonds, and therefore both could participate in thiol−disulfide interchange reactions (*23*). As a consequence, many more thiol−disulfide interaction pathways exist, and the separation and the analysis of the reaction products increase in complexity. Despite this, it appears that reactions between β-LG and κ-CN similar to those observed in the model systems occur in heated milk, although, as expected, other denatured whey proteins are also involved in complex formation (*11*, *15*, *16*, *24*−*29*).

Tools for the study of proteins and protein−protein interactions in complex systems have developed significantly in recent years with the introduction of proteomic tools, such as two-dimensional electrophoresis combined with mass spectrometry for the identification of specific separated proteins (*30*−*32*). A number of recent studies have applied two-dimensional electrophoretic separation approaches to the study of protein−protein interactions in heated milk systems, most often using sodium dodecyl sulfate−polyacrylamide gels under nonreducing and reducing conditions as the two dimensions for the separation of proteins, particularly disulfide-bonded complexes (*18*, *33*−*35*). Fewer studies have used the classic proteomic strategy of separation on the basis of isoelectric point (isoelectric focusing) followed by SDS-PAGE under reducing conditions (*36*), although this may yield information significantly different from that obtained through the previous approach mentioned.

The objective of this study was to investigate the interactions between proteins in raw skim milk and milk heated under laboratory conditions to identify the principal pathways and complexes involved, using proteomic approaches to protein separation and identification.

MATERIALS AND METHODS

Milk Samples and Reagents. Fresh bulk cow's milk was obtained from a dairy farm in Cork. The milk was warmed to 50 °C and skimmed using a pilot-scale cream separator (final fat content of < 0.1%, w/w). Sodium azide (0.02%, w/v) and a protease inhibitor cocktail, one tablet for 10 mL of milk (Complete Mini EDTA-free, Roche Diagnostics, Mannheim, Germany), were added to the fresh skim milk before analysis. Phosphoric and acetic acids were obtained from VWR (Ballycoolin, Dublin, Ireland); glycerol, urea, SDS, DTT, CHAPS, Triton X-100, iodoacetamide, bromophenol blue, and Coomassie blue were obtained from Sigma-Aldrich (St. Louis, MO); acrylamide was obtained from Bio-Rad (Hercules, CA).

Heat Treatment and Protein Preparation. Aliquots (2 mL) of skim milk were placed in glass tubes (1.5 cm diameter) with a parafilm cap and heated in a water bath at 90 °C for 30 min and then cooled rapidly in iced water and stored at −20 °C until analyzed. Following thawing, heat-treated milk samples were diluted (1/10) according to the method of Chevalier et al. (*37*) in 9 M urea, 4% CHAPS, w/v, and 0.05% Triton X-100, v/v, with (reduced samples) or without (unreduced samples) 65 mM DTT. The protein content of the solubilized samples was estimated using the Bradford method (*38*).

Analytical Two-Dimensional Electrophoresis. Precast 7-cm strips, pH range 4−7, or 17-cm strips, pH range 4−7 or 3−10 (Bio-Rad), as appropriate, were rehydrated in the presence of 100 μg (7-cm strips) or 300 μg (17-cm strips) of milk protein. Isoelectric focusing was carried out using a Protean IEF Cell (Bio-Rad) isoelectric focusing system until 50 kV h^{-1}. The strips were then incubated in the first equilibration solution [50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS] with 130 mM DTT (reduced samples) or without DTT (unreduced samples) and then in the second equilibration solution [50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS] with 130 mM iodoacetamide (reduced samples) or without iodoacetamide (unreduced samples). Strips were then embedded using 0.6% (w/v) low-melt agarose on the top of the acrylamide gel. SDS-PAGE was carried out on a 10−18% acrylamide gradient SDS−polyacrylamide gel (produced using a model 485 Gradient Former, Bio-Rad), using a Criterion Dodeca Cell electrophoresis unit (Bio-Rad). Gels were stained with colloidal Coomassie blue (*39*) and scanned to images, which were digitized with a GS 800 densitometer (Bio-Rad) and analyzed using PDQuest software v.7.3.1 (Bio-Rad).

Preparative Two-Dimensional Electrophoresis. Precast 17-cm strips pH 4−7 or pH 3−10 L (Bio-Rad) were rehydrated with 300 μg of protein samples, and isoelectric focusing was carried as described above. The strips were incubated in the first equilibrated solution with DTT (reduced samples) or without DTT (unreduced samples) and then in the SDS solution with iodoactamide. SDS-PAGE was carried out on linear 12.5% SDS−polyacrylamide gels, using the Criterion Dodeca Cell electrophoresis unit (Bio-Rad). Gels were stained with colloidal Coomassie blue (*39*), and images were digitized with a GS 800 densitometer (Bio-Rad) and analyzed using PDQuest software v.7.3.1 (Bio-Rad).

Image Analysis. An averaged gel experiment was performed with analytical two-dimensional electrophoresis gels using the gels of control unheated milk as reference with the PD Quest software (Bio-Rad). At least three experimental repetitions of each sample were used in this analysis. For each sample, the same gel areas were selected and compared. The spot volume was determined as percentage of total volume of all spots on respective gels.

MALDI-TOF and TOF/TOF Analysis. Spots were analyzed according to the method of Chevalier et al. (*40*). Briefly, spots were excised from preparative two-dimensional electrophoresis gels by hand and processed using a Packard Multiprobe II liquid-handling robot (Perkin-Elmer, Courtaboeuf, France). After successive washings with water, 25 mM ammonium bicarbonate, acetonitrile/25 mM ammonium bicarbonate (1:1, v/v), and acetonitrile, gel fragments were dried at 37 °C. Protein digestion was carried out at 37 °C for 5 h following addition of 0.125 μg of trypsin (sequencing grade, modified, Promega, Charbonières, France), and resulting fragments were extracted twice with 50 μL of acetonitrile/water (1:1, v/v) containing 0.1% trifluoroacetic acid for 15 min. Pooled supernatants were concentrated with a Speedvac to a final volume of ca. 20 μL. Peptides were simultaneously desalted and concentrated with C18 Zip-Tip microcolumns to a final volume of 3 μL, an aliquot of each sample was mixed (1/1) with the α-cyano-4-hydroxycinnamic acid matrix at half saturation in acetonitrile/water (1:1, v/v), and the mixture was immediately spotted on the MALDI target by the Multiprobe II robot. Mass spectra were recorded in the reflector mode on a UltraFlex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Automatic annotation of monoisotopic masses was performed using Bruker's SNAPTM procedure. The MASCOT search engine software (Matrix Science, London, U.K.) was used to search the NCBInr database. The following parameters were used: mass tolerance of 30 ppm, a minimum of five peptides matching to the protein, carbamidomethylation of cysteine as fixed modification, oxidation of methionine as variable modifications, and one missed cleavage allowed. MALDI-TOF/TOF experiments were performed under LID conditions with the LIFT cell voltage parameters set at 19.0 kV for a final acceleration of 29.5 kV (reflector voltage) and a pressure in the LIFT cell around 4×10^{-7} mbar. MASCOT parameters were adjusted as follows: parent ion mass tolerance of 20 ppm, fragment ion mass tolerance of 100 ppm.

Nano-LC MS Analysis. Stained protein spots were manually excised, washed, digested with trypsin as above, and extracted using formic acid instead of trifluoroactetic acid. Protein digests were analyzed using an ion trap mass spectrometer (Esquire HCT plus; Bruker Daltonics) coupled to a nanochromatography system (HPLC 1200, Agilent) interfaced with an HPLC-Chip system (Chip Cube, Agilent). MS/MS data were searched against National Center for Biotechnology Information (NCBI) and MSDB databases using Mascot software with the following parameters: parent and fragment ion mass tolerance of 0.6 Da for doubly and triply charged peptides.

RESULTS AND DISCUSSION

Reducing Two-Dimensional Electrophoresis Analysis of Raw Skim Milk Proteins: Reference Proteomic Map. A two-dimensional electrophoretogram of raw milk (**Figure 1**) highlighted the

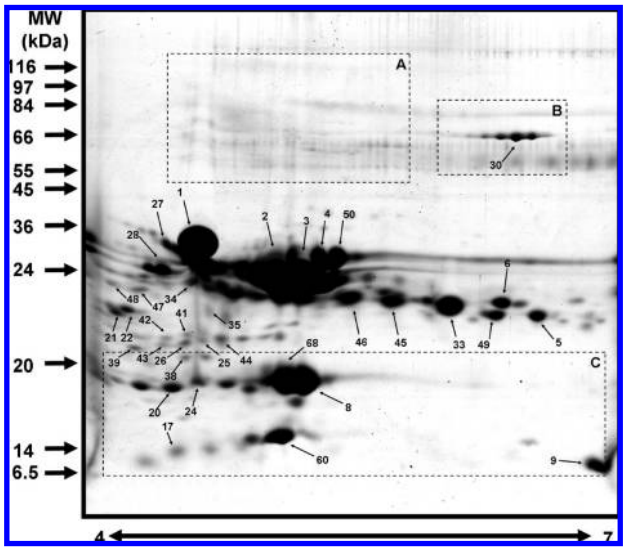

Figure 1. Two-dimensional gel electrophoretogram of proteins in raw milk (100 μg) separated under reducing conditions using a 7-cm pH 4−7 p*I* range strip for the first dimension and a 10−18% gradient acrylamide gel for the second dimension. The most abundant spots as indicated by arrows were submitted to mass spectrometry identification by MALDI-TOF peptide mass fingerprinting (**Table 1**). Three areas of interest indicated were compared according to heating time and reducing/nonreducing conditions of 2D separation (areas A, B, and C correspond to **Figures 4**, **5**, and **6**, respectively).

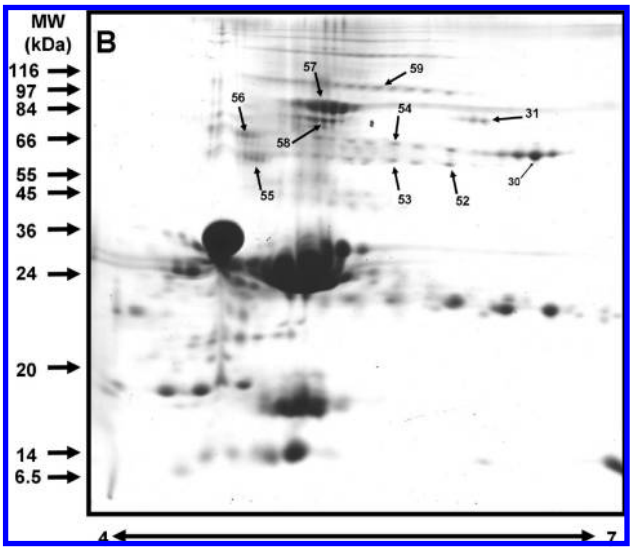

Figure 2. Two-dimensional gel electrophoretogram of proteins in raw milk (100 μg) separated under nonreducing conditions using a 7-cm pH 4−7 p*I* range strip for the first dimension and a 10−18% gradient acrylamide gel for the second dimension. The specific/interesting spots as indicated by arrows were submitted to mass spectrometry identification by MALDI-TOF peptide mass fingerprinting (**Table 1**).

complex heterogeneity of the milk protein system; this proteomic map of milk protein was prepared with 100 μg of reduced milk proteins using a wide p*I* range (4−7) for the first dimension and a gradient acrylamide gel (10−18%) for the second dimension (**Figure 1**). Between the two dimensions, the proteins were equilibrated with SDS in the presence of DTT followed by iodoacetamide, for reduction and alkylation of the proteins, respectively.

Despite the presence of dozens of proteins in milk identified using a global proteomic analysis, it is clear that the major proteins present were caseins (α_{S1}-, α_{S2}-, β-, κ-, and γ-), β-LG, α-lactalbumin (α-LA), and serum albumin, as would be expected (*41*) and which were found in positions roughly corresponding to those previously reported in similar two-dimensional analyses (*31*, *32*, *43*). Additional spots were clearly derived from these by alterations such as proteolysis, post-translational modification (e.g., spots 5, 6, 45, and 46 represent differently glycosylated forms of κ-CN), and interaction and association of the major milk proteins. For example, spot 9 is apparently a low molecular weight breakdown product of β-CN that has a very high isoelectric point, whereas spots 17, 20−22, 24−26, and 42−44 were all apparently proteolysis products of the caseins, probably originating from the activity of the principal indigenous milk protease, plasmin, or other indigenous (or exogenous, e.g., bacterial) protease activities in milk. Interestingly, there were far more proteolysis products originating from α_{S1}-CN than from β-CN and none from κ-CN, despite β-CN being the preferred substrate for plasmin.

Nonreducing Two-Dimensional Electrophoresis Analysis of Raw Skim Milk: Study of the Native Milk Interactome. When raw milk was subjected to two-dimensional analysis using nonreducing SDS-PAGE in the second dimension, a more complex pattern was obtained (**Figure 2**). By comparison to the reference proteomic map (**Figure 1**), the same separation parameters were applied to raw skim milk, but without the use of reducing agent, to prevent the reduction/cleavage of disulfide links between milk proteins: milk protein was prepared with 100 μg of unreduced milk proteins using a p*I* range (4−7) for the first dimension and a gradient acrylamide gel (10−18%) for the second dimension (**Figure 1**). Between the two dimensions, the proteins were equilibrated with SDS in the presence of iodoacetamide (without the DTT step) to fix the naturally reduced cysteines and to retain the naturally formed disulfide bonds. This strategy, which has been applied very recently and successfully to study the oxidative stress in chronic obstructive pulmonary disease (*42*), allowed observation of the naturally occurring disulfide interactions between the same proteins (homopolymers), and between different proteins (heteropolymers), in relation to the oxidized/reduced status of cysteine residues.

Comparison of the reducing and nonreducing milk proteomic maps highlighted significantly contrasting results (**Figure 2**). Most of the milk protein spots observed at molecular mass below 36 kDa were common to both conditions (bottom of the gel). In contrast, many high molecular weight spots appeared in the nonreducing proteomic map, showing new populations of disulfide-linked polymers (top of the gel). It can be noted that the spots observed in the reducing proteomic map between 14 and 36 kDa seemed to be less intense on the nonreducing proteomic map. As the same amount of protein was loaded for both conditions, the decrease in intensity of the low molecular weight resulted almost certainly from the presence of the disulfide-linked high molecular weight spots.

The use of a gel gradient (10−18%) for the second dimension of the two-dimensional electrophoresis was very powerful for the separation of these high molecular weight populations. At least 20 or 30 new spot populations were observed on the top of the gel of the nonreducing proteomic map. These spots were very heterogeneous in terms of isoelectic point (4.6−6.4) as well as molecular mass (45−150 kDa). The nine most intense spots of these disulfide-linked polymers were analyzed using mass spectrometry (**Table 1**, spots 31 and 52−59).

These complexes included some spots that may correspond to homopolymers of κ-CN (spots 52 and 53) and α_{S2}-CN (likely dimers at spots 57 and 58); these proteins, being the only CNs to contain cysteine residues, are known to form homomultimers (*43*).

Several heteropolymers consisting of various milk proteins at least in part disulfide-linked were also found, including complexes of α_{S2}/κ-CN (spot 54), α_{S1}-/α_{S2}/κ-CN (spots 55 and 56), α_{S1}-/α_{S2}/κ-CN/ β-Lg (spot 59), and α_{S2}/β-CN/serum albumin (spot 31), of different molecular weights and p*I* values, depending presumably on the glycosylated and phosphorylated forms of the CN involved. It can be noted that new proteins species were also identified in some of these complexes, including phenylethanolamine *N*-methyltransferase and α-actin 2 (spot 55), showing the complexity of the heteropolymeric complexes observed using such nonreducing two-dimensional electrophoresis. The observation of these stable complexes under "native-like" separation

Table 1. Principal Species Identified in Heated and Unheated Bovine Milk Samples by IEF/SDS-PAGE followed by (a) MALDI MS-PMF, (b) MALDI TOF/TOF, or (c) Nano-LC-MS/MS[a]

					MALDI-TOF (a)		MALDI-TOF/TOF (b)			nano-LC-MS/MS (c)	
spot	MW[b] theor/obsd	p*I* theor/ obsd[c]	identified protein	accession no.[d]	Mascot score	% coverage	peptide mass	Mascot score	sequence	score	matching peptides
1	22.97/27.3	4.91/4.61	α_{S1}-casein	P02662	84.4	31.8					
2	23.58/23.52	5.13/4.98	β-casein	P02666	39	15.6					
3	23.58/21.95	5.13/5.29	β-casein	P02666	52.4	19.2					
4	24.35/28.31	8.34/5.21	α_{S2}-casein	P02663	77.9	26.1				250	9
5	18.97/20.82	5.93/6.59	κ-casein	O02782	33	31.1					
6	18.97/21.26	5.93/6.34	κ-casein	O02782	31.9	27				139	2
8	18.28/18.27	4.83/5.16	β-lactoglobulin	P02754	55.7	44.4				461	10
9	23.58/14.2	5.13/6.95	β-casein	P02666	55.6	21.9				55	3
17	22.97/13.87	4.91/4.36	α_{S1}-casein	P02662	32	21.5					
20	23.58/17.92	5.13/4.46	β-casein	P02666			2061.81	44.9	FQSEEQQQ-TEDELQDK		
21	22.97/20.88	4.91/4.03	α_{S1}-casein	P02662	41.9	36.9					
22	22.97/20.75	4.91/4.12	α_{S1}-casein	P02662	42	26.2					
24	23.58/18.39	5.13/4.60	β-casein	P02666			2061.81	44.9	FQSEEQQQ-TEDELQDK		
25	22.97/19.93	4.91/4.61	α_{S1}-casein	P02662	69.8	24.3					
26	22.97/19.80	4.91/4.54	α_{S1}-casein	P02662	56.6	24.3					
27	22.97/31.09	4.91/4.34	α_{S1}-casein	P02662	61.2	31.8					
28	22.97/24.40	4.91/4.40	α_{S1}-casein	P02662	87.1	33.2					
30	66.43/65.11	5.6/6.39	serum albumin	P02769	85.3	16				923	19
31	24.35/70.32	8.34/6.18	α_{S2}-casein	P02663	49.1	18					
31	66.43/70.32	5.6/6.18	serum albumin	P02769	39.9	12.5					
31	23.58/70.32	5.13/6.18	β-casein	P02666	20.6	28.1					
33	18.97/21.15	5.93/6.01	κ-casein	O02782	28.7	30.5	1752.955	41.95	HPHPHLSFM-AIPPKK	198	3
34	22.97/21.80	4.91/4.56	α_{S1}-casein	P02662	51.5	24.3					
35	22.97/20.94	4.91/4.64	α_{S1}-casein	P02662	67.4	31.8					
38	23.58/19.22	5.13/4.54	β-casein	P02666			1029.54	36.35	HKEMPFPK		
39	22.97/19.33	4.91/4.18	α_{S1}-casein	P02662	41.5	16.8					
41	22.97/20.06	4.91/4.55	α_{S1}-casein	P02662	45.4	22.4					
42	22.97/19.92	4.91/4.44	α_{S1}-casein	P02662	51.5	24.3					
43	22.97/19.66	4.91/4.42	α_{S1}-casein	P02662	37.9	22.4					
44	22.97/19.95	4.91/4.71	α_{S1}-casein	P02662	54.5	22.4					
45	18.97/21.20	5.93/5.63	κ-casein	O02782	27.5	30.5					
46	18.97/21.28	5.93/5.38	κ-casein	O02782	27.9	42.4				102	3
47	24.35/21.53	8.34/4.26	α_{S2}-casein	P02663	35.4	15.7					
48	24.35/21.72	8.34/4.07	α_{S2}-casein	P02663	37.4	15.7					
49	18.97/20.74	5.93/6.19	κ-casein	O02782						40	1
50	24.35/28.94	8.34/5.32	α_{S2}-casein	P02663						264	7
52	18.97/55.27	5.93/5.98	κ-casein	O02782						171	3
53	18.97/56.66	5.93/5.63	κ-casein	O02782						120	1
54	18.97/61.37	5.93/5.65	κ-casein	O02782						120	2
54	24.35/61.37	8.34/5.65	α_{S2}-casein	P02663						83	4
55	41.77/56.38	5.24/4.81	α-actin 2	P62739						362	7
55	24.35/56.38	8.34/4.81	α_{S2}-casein	P02663						95	3
55	18.97/56.38	5.93/4.81	κ-casein	O02782						61	1
55	22.97/56.38	4.91/4.81	α_{S1}- casein	P02662						43	1
55	30.92/56.38	5.91/4.81	phenylethanol amine *N*-methyl-transferase	P10938						41	1
56	22.97/65.11	4.91/4.71	α_{S1}-casein	P02662						193	3
56	24.35/65.11	8.34/4.71	α_{S2}-casein	P02663						146	5
56	18.97/65.11	5.93/4.71	κ-casein	O02782						109	2
57	24.35/76.90	8.34/5.22	α_{S2}-casein	P02663	68.5	24.8					
57	24.35/76.90	8.34/5.22	α_{S2}-casein	P02663						251	7
58	24.35/70.85	8.34/5.21	α_{S2}-casein	P02663						112	2

Table 1. Continued

					MALDI-TOF (a)		MALDI-TOF/TOF (b)			nano-LC-MS/MS (c)	
spot	MW[b] theor/obsd	p*I* theor/ obsd[c]	identified protein	accession no.[d]	Mascot score	% coverage	peptide mass	Mascot score	sequence	score	matching peptides
59	18.28/89.38	4.83/5.55	β-lactoglobulin	P02754						202	4
59	22.97/89.38	4.91/5.55	α_{S1}- casein	P02662						181	3
59	18.97/89.38	5.93/5.55	κ-casein	O02782						175	3
59	24.35/89.38	8.34/5.55	α_{S2}-casein	P02663						140	4
60	14.19/14.73	4.8/5.04	α-lactalbumin	P00711						252	5
61	18.28/18.50	4.83/6.73	β-lactoglobulin	P02754						420	8
62	18.28/18.50	4.83/6.95	β-lactoglobulin	P02754						486	10
63	18.97/21.15	5.93/6.73	κ-casein	O02782						138	2
64	18.97/21.15	5.93/6.95	κ-casein	O02782						117	1
65	66.43/65.32	5.6/6.73	serum albumin	P02769						977	21
66	66.43/65.32	5.6/6.95	serum albumin	P02769						979	22
67	18.28/18.50	4.83/6.32	β-lactoglobulin	P02754						285	7
68	18.28/18.27	4.99/5.16	β-lactoglobulin	P02754						415	9

[a] Spot numbers correspond to **Figures 1**, **2**, **4**, **5**, **6**, and **7**. [b] Observed molecular mass from the gel (kDa)/theoretical molecular mass (kDa, calculated from the primary amino acid sequence using sequence analysis tools). [c] Observed isoelectric point from the gel/theoretical isoelectric point (calculated from the primary amino acid sequence using sequence analysis tools). [d] Accession number in the Swiss-Prot database.

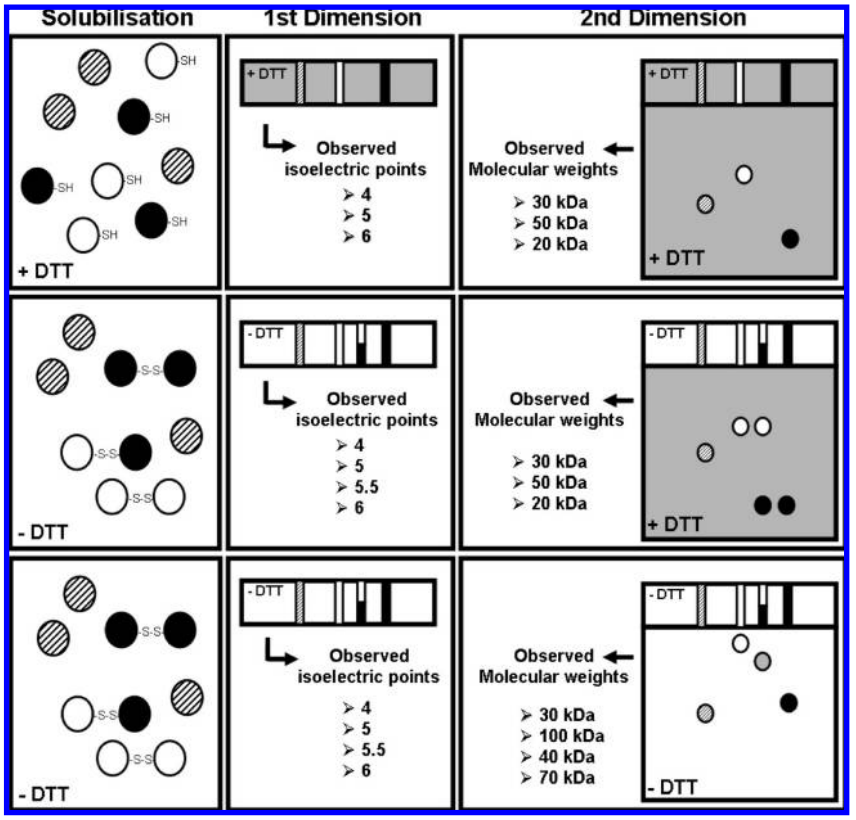

Figure 3. Schematic representation of the 2DE R/R, NR/R, and NR/NR strategies of analysis and the resulting expected results concerning the spots characteristic modification in term of isoelectric point and molecular weight. Milk samples were first diluted with solubilization buffer containing DTT (top panel, reduced samples) or without DTT (middle and bottom panel, unreduced samples) and then separated through 2D electrophoresis. Three samples conditions were set up to analyze and compare disulfide bridge exchanges (according to Miller et al. 2004): (a) samples completely reduced before and during 2-dimensional electrophoresis (top panel); (b) samples unreduced before 2-dimensional electrophoresis and reduced only after isoelectric focusing (middle panel); (c) samples unreduced before and during 2-dimensional electrophoresis (bottom panel).

conditions (i.e., without ionic detergents or reducing agents) may reveal natural interactions between milk proteins with a potential biological function, still unknown. The interaction of whey proteins (i.e., especially β-LG) and caseins (α_{S1}-/α_{S2}/κ-/β-CN) at the micellar surface can explain, at least in part, the initiation of a three-dimensional gel matrix during ultrahigh-temperature treatment involving whey proteins and caseins (*44*).

Nonreducing versus Reducing Two-Dimensional Electrophoresis of Heated Milk: Analysis of Heat-Induced Interactions. The changes in milk during heating at 90 °C for up to 30 min were analyzed by two-dimensional electrophoresis under three different sets of conditions of treatment (the principle is schematically described in **Figure 3**): 2DE R/R corresponds to samples analyzed under reducing conditions in both dimensions (i.e., all disulfide bonds were reduced during the full analysis, and complexes were resolved into their constituent proteins); 2DE NR/R corresponds to samples reduced only after isoelectric focusing (i.e., disulfide-bonded complexes resolved during IEF, but the bonds were

reduced during SDS-PAGE); and 2DE NR/NR corresponds to fully unreduced samples (i.e., disulfide-bonded complexes remained cross-linked throughout both dimensions of analysis). This strategy of analysis was first described by Miller et al. (*45*) for the specific study of disulfide-bond-mediated interactions between urinary proteins. This strategy, thanks to the powerful precision of IEF, represents a more accurate alternative to the strategy of consecutive steps of SDS-PAGE under nonreducing and reducing conditions (*33*). Analysis under R/R conditions corresponds to the conventional technique used to separate a complex mixture of reduced proteins through two-dimensional electrophoresis and was used to prepare the raw skim milk proteomic reference map (**Figure 1**), whereas analysis under NR/NR conditions was used to study the disulfide-bonded interactome in raw milk (**Figure 2**).

Samples of raw skimmed milk after heating for 0, 5, 10, and 30 min at 90 °C were submitted to these three different conditions of 2DE in triplicate experiments; the resulting gels were analyzed and spots of interest were identified using tryptic digestion followed by mass spectrometry, that is, MALDI-TOF mass spectra. Three areas of particular interest were selected and are highlighted in **Figure 1** (A, B, and C).

Area A corresponds to high molecular weight proteins or complexes (**Figure 4**), which were almost all observed only when 2-DE was carried out under completely nonreducing conditions (2DE NR/NR); consequently, these complexes were clearly disulfide-linked. After identification of the spots by MALDI-TOF MS, β-LG, κ-CN, β-CN, and α_{S1}-CN were identified as being involved in some of these spots. The former proteins are well-known to undergo heat-induced complex formation through thiol−disulfide interchange reactions (*19*, *46*), but the participation of the other two caseins determined is less widely reported, showing the capacity of this analysis strategy to identify constituent proteins in complex polymers. Under complete (R/R) or partial (NR/R) reducing conditions, only spot 56 can be matched with the NR/NR conditions for both heated times. This spot corresponds to a mixture of α_{S1}-/α_{S2}-/κ-CN. This heteropolymer was naturally observed under "native" nonreducing 2DE of raw milk and seemed to be partially resistant to reduction after heat treatment at 90 °C. It can be hypothesized that such high temperature can denature proteins and complexes and consequently hide disulfide bonds inside the three-dimensional structures, protecting them in part from reduction by DTT. Such high temperatures can also increase the glycation reactions between proteins and lactose and can induce covalently linked complexes, resistant to reducing agents (*47*). These oxidized complexes included disulfide bridges and a possible formation of a protein cross-link from initial Amadori adducts of lactose and ε-lysyl residues involving an imidazole function (*48*, *49*). Proteins without cysteine residues (β-CN and α_{S1}-CN) were possibly linked to these complexes by sugar-type bonds catalyzed by a nonenzymatic glycation process.

Spots in area B, which corresponds to several isoforms (spot 30) of bovine serum albumin (BSA), were also compared by heat treatment time and conditions of separation (**Figure 5**). These spots were observed in the control (unheated milk) sample under reducing and nonreducing conditions. However, when the milk was heated at 90 °C, the spots disappeared when IEF was carried out under nonreducing conditions. Thus, it appeared that BSA readily formed heat-induced complexes of high molecular weight which could not be resolved unless DTT was present in the initial stage of electrophoresis. BSA contains one free sulfhydryl of its 35 cysteines and is consequently highly susceptible to denaturation and aggregation on heating (*3*).

Area C (**Figure 6**) contained spots corresponding to the major whey proteins, β-LG (spots 8 and 68) and α-LA (spot 60). The two major genetic variants of β-LG (A and B) were identified by mass spectrometry as β-LG, but without any additional information concerning the nature of the analyzed variant. β-LG variants A and B differ only by two amino acids in position 80 (D/G) and 134 (D/A), respectively, according to the Swiss-Prot database

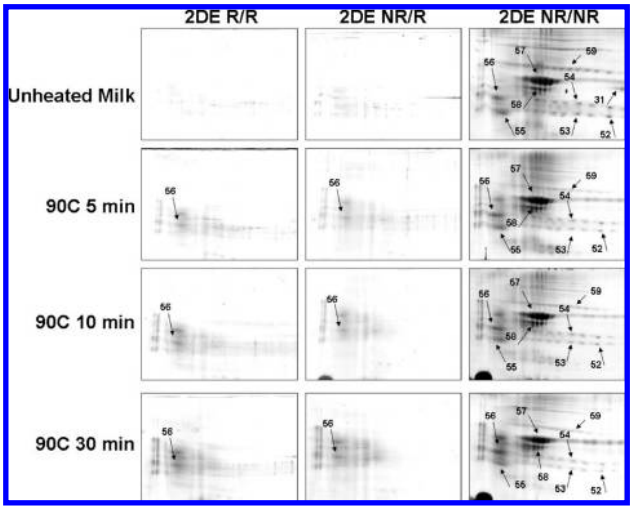

Figure 4. Comparison of changes in spots in area A of **Figure 1** as a function of heating time and different conditions of 2D electrophoresis.

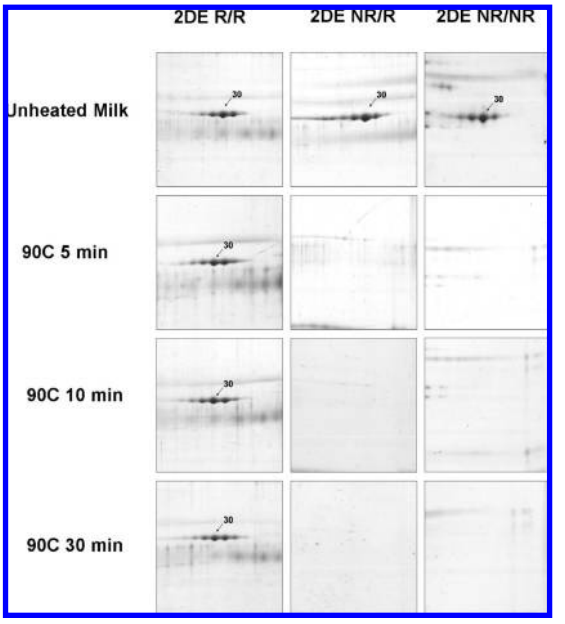

Figure 5. Comparison of changes in spots in area B of **Figure 1** as a function of heating time and reducing/nonreducing conditions of 2D electrophoresis.

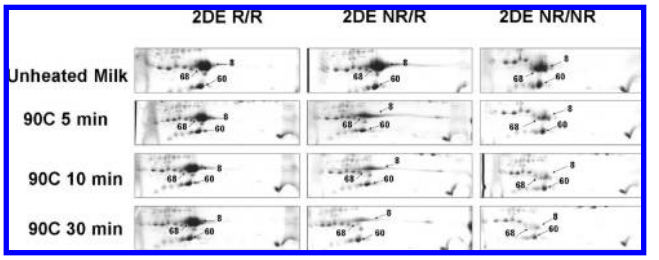

Figure 6. Comparison of changes in spots in area C of **Figure 1** as a function of heating time and reducing/nonreducing conditions of 2D electrophoresis.

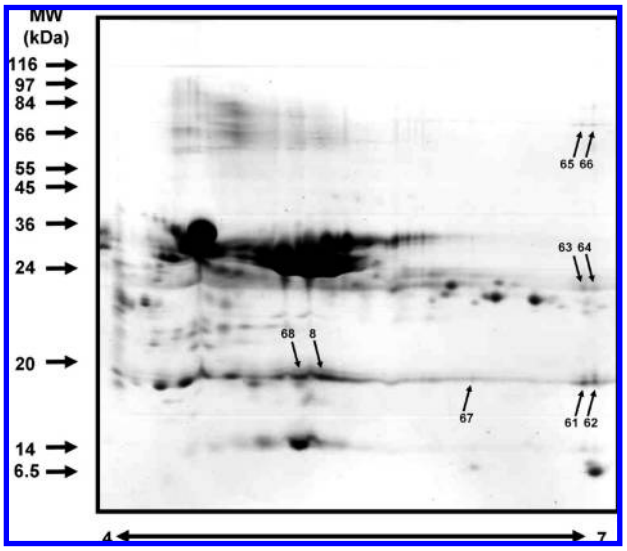

Figure 7. Two-dimensional gel electrophoretogram of milk protein (100 μg) separated under nonreducing conditions of solubilization and isoelectric focusing and then reducing condition of SDS-PAGE second dimension, using a 7-cm pH 4−7 p*I* range for the first dimension and a 10−18% gradient acrylamide gel for the second dimension. The specific/interesting spots are shown with arrows and were submitted to mass spectrometry identification by MALDI-TOF peptide mass fingerprint (**Table 1**).

(http://expasy.org/sprot), using the β-LG B accession number (P02754) as reference. From the primary sequence of the protein, the theoretical isoelectric point is 4.83 for β-LG variant B and 4.76 for variant A. Consequently, we can reasonably propose to identify spot 68 as variant A and spot 8 as variant B. These two variants of β-LG (A and B) were apparent in control samples and in heated milk samples analyzed under completely reducing conditions (top three panels and left four panels of **Figure 6**). Under completely nonreducing conditions, however, the intensity of the two protein spots decreased progressively and, after 30 min at 90 °C, the spots had almost completely disappeared. Over the same time, spot 60, corresponding to α-LA, showed the same pattern as β-LG's spots, with no intensity changes under 2DE R/R conditions, but with a progressive decrease of spot intensity under NR/R and NR/NR conditions as a function of heating time, showing that a part of α-LA was also involved in disulfide-linked polymers, even if no α-LA was identified in such oxidized complexes.

Part of the disulfide-cross-linked complex of β-LG could be reduced when DTT was added during the second dimension (NR/R), but with a range of isoelectric points from 5 to 7, revealed by a nonfocused smear on the right of the β-LG spots 8 and 68. The disulfide-bonded complexes of β-LG showed a large heterogeneity of isoelectric points, presumably due to the high molecular weights of polymers, which were difficult to focus normally during the first nonreducing dimension of the 2DE.

To further understand the protein species involved in these complexes, a preparative gel was prepared under NR/R reducing conditions using a milk sample heated for 30 min at 90 °C (**Figure 7**). On the basis of molecular weight, β-LG was identified in spots 61, 62, 67, and 68; κ-CN in spots 63 and 64; and serum albumin in spots 65 and 66. In contrast, the isoelectric point of the identified protein is far from the theoretical isoelectric point. The reducing conditions for the second dimension allowed determination of the correct molecular weight of all of these proteins. Nevertheless, the nonreducing condition of the first dimension did not result in dissociation of the complexes formed with disulfides bridges. This condition clearly showed a heterotrimer complex between one or more molecules of β-LG, κ-CN, and BSA formed by disulfide bonding, which focused near pH 7, the isoelectric point of spots 61−66. It can also be hypothesized that spot 67, which corresponds to β-LG, was involved in a complex of the same origin but involving other proteins. Although these complexes were clearly observed using nonreducing versus reducing 2DE conditions, the disulfide bridges involved in the homo/heteropolymer formation is still unknown. The next step of the present study will be the identification and localization of the disulfide bridges, as previously performed on goat's milk (*50*).

Conclusions. The strategy of separation selected for this study showed new findings on the "native-like" disulfide interactions between milk proteins, with or without any heat treatments. The proteomic tools resolved clearly a complex mixture of proteins in raw milk, including some natural disulfide-linked complexes; heating milk fundamentally altered the milk proteome and resulted in a range of cross-linked protein adducts, many of which were identified by mass spectrometry. In this study, it was shown that different two-dimensional electrophoresis separations followed by mass spectrometry have the capacity to identify the protein partners involved inside oxidized complexes, which can potentially be used as potential markers of milk oxidation (*51*). The reduced or oxidized state of proteins in a very complex environment of reducing sugar and lipids such as milk can be an indicator of the quality and storage capacity of dairy products.

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LITERATURE CITED

(1) Sawyer, W. H. Complex between β-lactoglobulin and κ-casein. A review. *J. Dairy Sci.* **1969**, *52*, 1347–1355.
(2) Gezimati, J.; Creamer, L. K.; Singh, H. Heat-induced interactions and gelation of mixtures of β-lactoglobulin and alpha-lactalbumin. *J. Agric. Food Chem.* **1997**, *45*, 1130–1136.
(3) Havea, P.; Singh, H.; Creamer, L. K. Characterization of heat-induced aggregates of β-lactoglobulin, α-lactalbumin and bovine serum albumin in a whey protein concentrate environment. *J. Dairy Res.* **2001**, *68*, 483–497.
(4) Bertrand-Harb, C.; Baday, A.; Dalgalarrondo, M.; Chobert, J.- M.; Haertle, T. Thermal modifications of structure and co-denaturation of α-lactalbumin and β-lactoglobulin induce changes of solubility and susceptibility to proteases. *Nahrung* **2002**, *46*, 283–289.
(5) Cho, Y.; Singh, H.; Creamer, L. K. Heat-induced interactions of β-lactoglobulin A and κ-casein B in a model system. *J. Dairy Res.* **2003**, *70*, 61–71.
(6) Zittle, C. A.; Thompson, M. P.; Custer, J. H.; Cerbulis, J. κ-Casein−β-lactoglobulin interaction in solution when heated. *J. Dairy Sci.* **1962**, *45*, 807–810.
(7) Sawyer, W. H.; Coulter, S. T.; Jenness, R. Role of sulfhydryl groups in the interaction of κ-casein and β-lactoglobulin. *J. Dairy Sci.* **1963**, *46*, 564–565.
(8) McKenzie, H. A. α-Lactoglobulins. In *Milk Proteins: Chemistry and Molecular Biology*; McKenzie, H. A., Ed.; Academic Press: New York, 1971; pp 257−330.
(9) Hill, A. R. The β-lactoglobulin−κ-casein complex. *Can. Inst. Food Sci. Technol. J.* **1989**, *22*, 120–123.
(10) Jang, H. D.; Swaisgood, H. E. Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. *J. Dairy Sci.* **1990**, *73*, 900–904.
(11) Corredig, M.; Dalgleish, D. G. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. *Food Res. Int.* **1996**, *29*, 49–55.
(12) Corredig, M.; Dalgleish, D. G. The binding of α-lactalbumin and β-lactoglobulin to casein micelles in milk treated by different heating systems. *Milchwissenschaft* **1996**, *51*, 123–127.

(13) Corredig, M.; Dalgleish, D. G. The mechanisms of the heatinduced interaction of whey proteins with casein micelles in milk. *Int. Dairy J.* **1999**, *9*, 233–236.
(14) Euber, J. R.; Brunner, J. R. Interaction of κ-casein with immobilized β-lactoglobulin. *J. Dairy Sci.* **1982**, *65*, 2384–2387.
(15) Anema, S. G.; Li, Y. Effect of pH on the association of denatured whey proteins with casein micelles in heated reconstituted skim milk. *J. Agric. Food Chem.* **2003**, *51*, 1640–1646.
(16) Anema, S. G.; Li, Y. Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size. *J. Dairy Res.* **2003**, *70*, 73–83.
(17) McKenzie, G. H.; Norton, R. S.; Sawyer, W. H. Heat-induced interaction of β-lactoglobulin and κ-casein. *J. Dairy Res.* **1971**, *38*, 343–351.
(18) Creamer, L. K.; Bienvenue, A.; Nilsson, H.; Paulsson, M.; van Wanroij, M.; Lowe, E. K.; Anema, S. G.; Boland, M. J.; Jiménez-Flores, R. Heat-induced redistribution of disulfide bonds in milk proteins. 1. Bovine α-lactoglobulin. *J. Agric. Food Chem.* **2004**, *52*, 7660–7668.
(19) Lowe, E. K.; Anema, S. G.; Bienvenue, A.; Boland, M. J.; Creamer, L. K.; Jimenez-Flores, R. Heat-induced redistribution of disulfide bonds in milk proteins. 2. Disulfide bonding patterns between bovine β-lactoglobulin and κ-casein. *J. Agric. Food Chem.* **2004**, *52*, 7669–7680.
(20) Jang, H. D.; Swaisgood, E. Disulphide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. *J. Dairy Sci.* **1990**, *73*, 900–904.
(21) Livney, Y. D.; Dalgleish, D. G. Specificity of disulphide bond formation during thermal aggregation in solutions of β-lactoglobulin B and κ-casein A. *J. Agric. Food Chem.* **2004**, *52*, 5527–5532.
(22) Swaisgood, H. E. Chemistry of the caseins. In *Advanced Dairy Chemistry. s1. Proteins*; Fox, P. F., Ed.; Elsevier Applied Science: London, U.K., 1992; pp 63−110.
(23) Rasmussen, L. K.; Johnsen, L. B.; Tsiora, A.; Sørensen, E. S.; Thomsen, J. K.; Nielsen, N. C.; Jakobsen, H. J.; Petersen, T. E. Disulphide-linked caseins and casein micelles. *Int. Dairy J.* **1999**, *9*, 215–218.
(24) Guyomarc'h, F.; Law, A. J. R.; Dalgleish, D. G. Formation of soluble and micelle-bound protein aggregates in heated milk. *J. Agric. Food Chem.* **2003**, *51*, 4652–4660.
(25) Smits, P.; van Brouwershaven, J. H. Heat-induced association of β-lactoglobulin and casein micelles. *J. Dairy Res.* **1980**, *47*, 313–325.
(26) Singh, H.; Creamer, L. K. Denaturation, aggregation and heat stability of milk protein during the manufacture of skim milk powder. *J. Dairy Res.* **1991**, *58*, 269–283.
(27) Vasbinder, A. J.; de Kruif, C. G. Casein−whey protein interactions in heated milk: the influence of pH. *Int. Dairy J.* **2003**, *13*, 669–677.
(28) Nabhan, M. A.; Girardet, J. M.; Campagna, S.; Gaillard, J. L.; Le Roux, Y. Isolation and characterization of copolymers of β-lactoglobulin, α-lactalbumin, κ-casein, and α_{S1}-casein generated by pressurization and thermal treatment of raw milk. *J. Dairy Sci.* **2004**, *87*, 3614–3622.
(29) Oldfield, D. J.; Singh, H.; Taylor, M. W. Kinetics of heat-induced whey protein denaturation and aggregation in skim milks with adjusted whey protein concentration. *J. Dairy Res.* **2005**, *72*, 369–378.
(30) Galvani, M.; Hamdan, M.; Righetti, P. G. Two-dimensional gel electrophoresis/matrix-assisted laser desorption/ionization mass spectrometry of a milk powder. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1889–1897.
(31) Holland, J. W.; Deeth, H. C.; Alewood, P. F. Proteomic analysis of κ-casein micro-heterogeneity. *Proteomics* **2004**, *4*, 743–752.
(32) Holland, J. W.; Deeth, H. C.; Alewood, P. F. Resolution and characterisation of multiple isoforms of bovine κ-casein by 2-DE following a reversible cysteine-tagging enrichment strategy. *Proteomics* **2006**, *6*, 3087–3095.
(33) Patel, H. A.; Singh, H.; Anema, S. G.; Creamer, L. K. Effects of heat and high hydrostatic pressure treatments on disulphide bonding interchanges among the proteins in skim milk. *J. Agric. Food Chem.* **2006**, *54*, 3409–3420.
(34) Creamer, L. K.; Bienvenue, A.; Nilsson, H.; Paulsson, M.; van Wanroij, M.; Lowe, E. K.; Anema, S. G.; Boland, M. J.; Jiménez-Flores, R. Heat-induced redistribution of disulphide bonds in milk proteins. 1. Bovine β-lactoglobulin. *J. Agric. Food Chem.* **2004**, *52*, 7660–7668.
(35) Livney, Y. D.; Dalgleish, D. G. Specificity of disulfide bond formation during thermal aggregation in solutions of β-lactoglobulin B and κ-casein A. *J. Agric. Food Chem.* **2004**, *52*, 5527–5532.
(36) Yamada, M.; Murakami, K.; Wallingford, J. C.; Yuki, Y. Identification of low-abundance proteins of bovine colostral and mature milk using two-dimensional electrophoresis followed by microsequencing and mass spectrometry. *Electrophoresis* **2002**, *23*, 1153–1160.
(37) Chevalier, F.; Martin, O.; Rofidal, V.; Devauchelle, A. -D.; Rossignol, M. Proteomic investigation of natural variation between *Arabidopsis* ecotypes. *Proteomics* **2004**, *4*, 1372–1381.
(38) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(39) Chevalier, F.; Rofidal, V.; Vanova, P.; Bergoin, A.; Rossignol, M. Proteomic capacity of recent fluorescent dyes for protein staining. *Phytochemistry* **2004**, *65*, 1499–1506.
(40) Chevalier, F.; Centeno, D.; Rofidal, V.; Tauzin, M.; Sommerer, N.; Rossignol, M. Differential capacity of visible stains and fluorescent dyes for large-scale investigation of proteomes by MALDI-TOF MS. *J. Proteome Res.* **2006**, *5*, 512–520.
(41) Fox, P. F.; McSweeney, P. L. H. *Dairy Chemistry and Biochemistry*; Blackie Academic and Professional Publishers: London, U.K., 1998.
(42) Lehtonen, S. T.; Ohlmeier, S.; Kaarteenaho-Wiik, R.; Harju, T.; Pääkkö, P.; Soini, Y.; Kinnula, V. L. Does the oxidative stress in chronic obstructive pulmonary disease cause thioredoxin/peroxiredoxin oxidation? *Antioxid. Redox. Signal.* **2008**, *10*, 813–819.
(43) Holland, J. W.; Deeth, H. C.; Alewood, P. F. Analysis of disulphide linkages in bovine κ-casein oligomers using two-dimensional electrophoresis. *Electrophoresis* **2008**, *29*, 2402–2410.
(44) Datta, N.; Deeth, H. C. Age gelation of UHT milk: a review. *Food Bioprod. Proc.* **2001**, *79*, 197–210.
(45) Miller, I.; Teinfalt, M.; Leschnik, M.; Wait, R.; Gemeiner, M. Non-reducing two-dimensional gel electrophoresis for the detection of Bence Jones proteins in serum and urine. *Proteomics* **2004**, *4*, 257–260.
(46) Considine, T.; Patel, H. A.; Anema, S. G.; Singh, H.; Creamer, L. K. Interactions of milk proteins during heat and high hydrostatic pressure treatments—a review. *Innovative Food Sci. Emerg. Technol.* **2007**, *8*, 1–23.
(47) Chevalier, F.; Chobert, J.-M.; Mollé, D.; Haertlé, T. Maillard glycation of β-lactoglobulin with several sugars: comparative study of the properties of the obtained polymers and of the substituted sites. *Lait* **2001**, *81*, 655–666.
(48) Chevalier, F.; Chobert, J.-M.; Dalgalarrondo, M.; Haertlé, T. Characterization of the Maillard reactions product of β-lactoglobulin glucosylated in mild conditions. *J. Food Biochem.* **2001**, *25*, 33–55.
(49) Pongor, S.; Ulrich, P. C.; Bencsath, F. A.; Cerami, A. Aging of proteins: isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2684–2688.
(50) Henry, G.; Mollé, D.; Morgan, F.; Fauquant, J.; Bouhallab, S. Heat-induced covalent complex between casein micelles and β-lactoglobulin from goat's milk: identification of an involved disulfide bond. *J. Agric. Food Chem.* **2002**, *50*, 185–191.
(51) Wait, R.; Begum, S.; Brambilla, D.; Carabelli, A. M.; Conserva, F.; Rocco Guerini, A.; Eberini, I.; Ballerio, R.; Gemeiner, M.; Miller, I.; Gianazza, E. Redox options in two-dimensional electrophoresis. *Amino Acids* **2005**, *28*, 239–272.

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