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Proteomic Quantification of Disulfide-Linked Polymers in Raw and Heated Bovine Milk

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Disulfide bond formation between milk protein molecules was quantified in raw and heated bovine milk using reducing and nonreducing two-dimensional electrophoresis. Analysis of protein profiles in raw milk indicated that 18% of α_{S2}-casein, 25% of β-lactoglobulin, and 46% of κ-casein molecules were involved in disulfide-linked complexes (calculated through differences in spot volumes on two-dimensional electrophoretograms under reducing and nonreducing conditions), whereas levels of α_{S1}- and β-caseins were similar under both conditions. Following heat treatment at 90 °C for 30 min, spot volumes of serum albumin, β-lactoglobulin, and κ-casein decreased by 85%, 75%, and 75%, respectively, with the formation of several spots on nonreducing gels corresponding to polymers. Homopolymers and heteropolymers of κ-casein and α_{S2}-casein were identified by mass spectrometry in raw milk samples; polymers involving only α_{S2}-casein or only κ-casein accounted for 43% and 12% of the total polymers present, respectively. In addition, 45% of polymers in raw milk involved α_{S2}-casein in association with other proteins as heteropolymers, indicating the key role of this protein in intermolecular disulfide bridging between proteins in raw milk. The intensity of monomeric κ-casein spots decreased progressively with heating time at 90 °C, with greatest changes in spots with acidic isoelectric points. Interactions and association of milk proteins via disulfide bridges are discussed in relation to the proteins involved and their potential protective function against formation of fibril aggregates.

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

INTRODUCTION

One of the major objectives of dairy biochemistry is understanding of functional and biological properties of biomolecules. For this purpose, the techniques most widely used to visualize and analyze proteins are SDS−PAGE and two-dimensional electrophoresis (2DE) under denaturing conditions. Milk proteins have been analyzed extensively using proteomic tools (*1*−*13*). Based on two independent biochemical characteristics of proteins, 2DE combines isoelectric focusing (IEF), which separates proteins according to their isoelectric point (related to amino acid characteristics, i.e., acid/basic/neutral), and SDS−PAGE, which separates them further according to their molecular mass. At present, 2DE allows simultaneous detection and quantification of up to thousands of protein spots in the same gel (*14*, *15*). Combined with subsequent mass spectrometry analysis, 2DE offers the possibility of identifying proteins and comparing isoform abundance and posttranslational modifications (*16*−*19*).

Despite numerous proteomic studies analyzing milk proteins, many questions concerning milk protein expression, modification, and interactions remain unanswered. Milk is a very complex fluid secretion, composed of a colloidal emulsion of fat globules and casein micelles in a water-based fluid containing soluble proteins, lactose, and many trace elements such as vitamins and minerals (*20*). In this biological system, caseins are organized and stabilized in micelles (*21*−*23*). The biological function of the caseins is largely nutritional; casein micelles serve as calcium-transport vesicles, providing young mammals with a concentrated, yet soluble, form of calcium as well as essential amino acids (*24*). Another biological function of caseins was described recently (*25*), where these proteins can act as chaperones to protect proteins against aberrant aggregations, including formation of amyloid fibrils, when exposed to stress conditions such as elevated temperature or reducing environment (*26*−*29*).

During processing, bovine milk is submitted to several physical treatments, especially homogenization and pasteurization. These processes are well-known to induce modifications in protein organizations (*30*−*32*). Heat treatment of milk results in a number of physicochemical changes in the milk constituents, in particular, denaturation of whey proteins, leading to hydrophobic interactions or disulfide-bonded aggregation with κ-casein at the surface of casein micelles (*31*−*35*). Two disulfide bridges and a free sulfhydryl group present in the native structure of the whey protein β-lactoglobulin seem to play a critical role in its heat-induced interactions with κ-casein (*36*−*42*). Patel et al. (*43*) recently developed a system based on a two-dimensional PAGE method to explore the differences in the irreversible disulfide bond changes among the milk proteins after heat pressure treatments. They

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observed disulfide-bonded aggregates that included a high proportion of all the whey proteins and κ-casein and a proportion of the α_{S2}-casein. Following these important novel findings, a similar method to analyze milk protein complexes, based on the strategy of Miller et al. (*44*), using a combination of nonreducing and reducing steps with a high resolutive conventional proteomic approach (*45*), was developed. Nonreducing IEF was first performed to separate disulfide-linked polymers, and then reducing or nonreducing SDS−PAGE was used as a second dimension to separate protein complexes. Protein spots were analyzed by mass spectrometry to identify the proteins involved in each complex.

Based on the same analysis strategy, in the present study, the complexes formed under native-like conditions or after heat treatment were characterized, quantified, and compared with the corresponding nonaggregated protein spots to understand a possible biological function of such interactions. Following the approach of Holland et al. (*46*), a special focus was placed on κ-casein spots to understand and quantify the milk proteins which preferentially interact in the native environment or after heat treatment.

MATERIALS AND METHODS

Milk Treatment and Protein Solubilization. Fresh raw milk was obtained, skimmed, and preserved as described in ref *45*; subsamples of skim milk were also heated in a water bath at 90 °C for time periods up to 30 min. Samples were then prepared for electrophoresis with or without reducing agent, and protein content was measured as described in ref *45*.

One-Dimensional Gel Electrophoresis. Separation of milk samples was carried out according to the method of Laemmli (*47*) on 12.5% acrylamide gels using the Protean II system (Bio-Rad, Hercules, CA). The gels were stained with Coomassie Blue (R250) and scanned to digital images with a GS-800 densitometer (Bio-Rad).

Two-Dimensional Gel Electrophoresis. Each milk sample was analyzed using two-dimensional gel electrophoresis as described previously (*45*). Briefly, precast 7 cm strips, pH range 4−7 (Bio-Rad, Hercules, CA), were rehydrated in the presence of 100 μg of reduced or unreduced milk protein. Isoelectric focusing was carried out using a Protean IEF cell (Bio-Rad, Hercules, CA) until 50 kV h^{-1} and submitted to the second dimension. SDS−PAGE was carried out on a 10−18% acrylamide gradient (Gradient former 485; Bio-Rad) using a Criterion Dodeca Cell electrophoresis unit (Bio-Rad). Gels were stained with colloidal Coomassie Blue (*48*) and scanned to images with a GS 800 densitometer (Bio-Rad). Two-dimensional gels were analyzed with the Progenesis Samespots software V3.3 (Nonlinear). The same gel area was selected and compared, and the spot volume was quantified as a mean of each replicate. For each picture used, the same spots were reproduced, and when no spot was detected, a background value was automatically created by the software and used in the analysis. All spots and isoforms were detected, and spot volumes were compared between samples, and apparent isoelectric points and molecular weights were calculated from spot localization in the 2DE maps.

Mass Spectrometry Analysis. Stained protein spots were excised manually, washed, digested with trypsin as previously described (*45*), and extracted using formic 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 NCBI (National Center for Biotechnology Information) and MSDB databases using Mascot software with a parent and fragment ion mass tolerance of 0.6 Da for doubly and triply charged peptides.

RESULTS

Electrophoretic Analysis (One-Dimensional) of Raw and Heated Skim Milk. Separation of milk proteins using SDS−PAGE is a conventional technique to visualize the different protein species in a given sample; electrophoretic separation of proteins in the milk sample is shown in **Figure 1**. Under reducing conditions (**Figure 1**, left part), BSA, κ-casein, β-lactoglobulin, and α-lactalbumin were well separated. However, α_{S1}-, α_{S2}-, and β-caseins were badly separated owing to their very similar molecular weights. No modification of the protein pattern under reducing condition was observed between the raw milk sample (**Figure 1**, lane 1) and samples heated for 5, 10, or 30 min at 90 °C (**Figure 1**, lanes 2, 3, and 4, respectively).

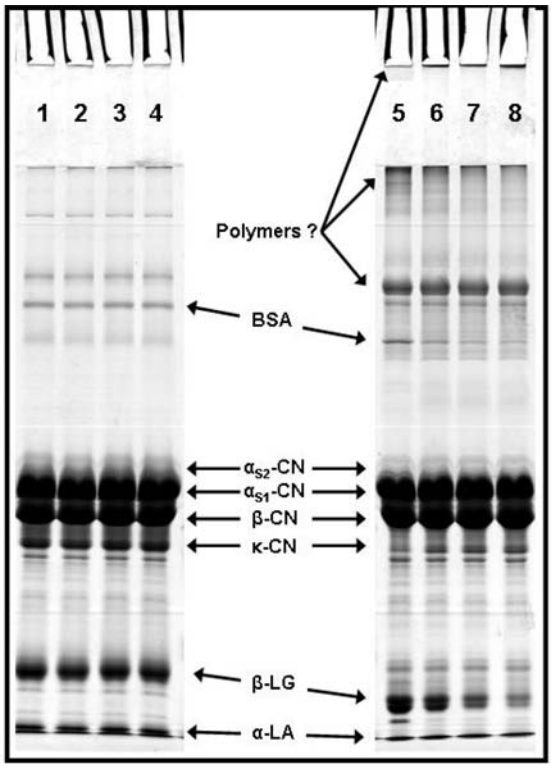

Figure 1. SDS−PAGE electropherogram of raw milk (lanes 1 and 5) or milk heated for 5 min (lanes 2 and 6), 10 min (lanes 3 and 7), or 30 min (lanes 4 and 8) at 90 °C under reducing (left part) and nonreducing (right part) conditions.

Under nonreducing conditions (SDS−PAGE without β-mercaptoethanol) some differences can be observed. Compared to reduced monomeric κ-casein, the amount of unreduced protein was considerably decreased in raw milk (**Figure 1**, lane 5) and remained at the same level during heat treatment, whereas amounts of unreduced BSA and β-lactoglobulin bands disappeared progressively during heat treatment (**Figure 1**, lanes 6−8), with concomitant appearance of polymers in the upper part of the gel. These polymers were expected to be formed by the interaction of κ-casein, β-lactoglobulin, and other milk proteins containing available sulfhydryl groups. The reactivity of milk proteins in forming polymers has been largely described using model systems. However, many questions concerning the composition and the amount of polymers cannot be answered owing to the low separation power of SDS−PAGE. Thus, a new strategy based on proteomic analysis, using high-resolution IEF followed by SDS−PAGE, was developed (*45*).

Comparison of Nonreducing and Reducing 2DE of Proteins in Raw Milk. Two-dimensional electrophoresis of proteins in unheated milk was performed under reducing and nonreducing conditions (**Figure 2**). The proteomic map obtained under reducing conditions was characteristic of a milk sample. Several spots for each casein and whey protein were observed and identified using mass spectrometry (**Table 1**). The use of IEF as the first dimension resolved multiple protein isoforms in comparison with the 1D analysis (right part of the 2D gel), without IEF. Most of the κ-casein spots were easily observed and corresponded to

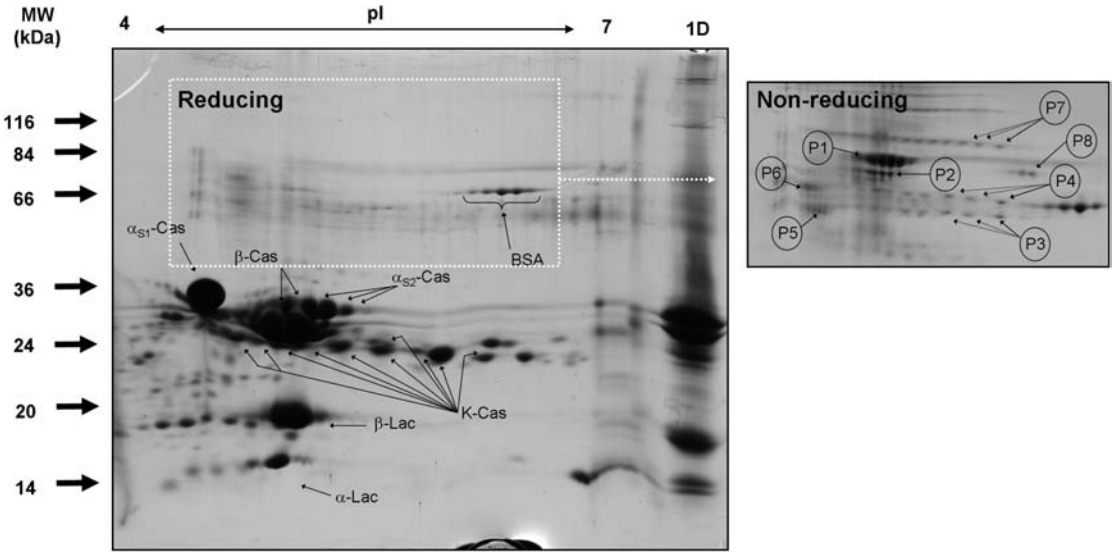

Figure 2. Two-dimensional electropherogram of proteins in raw milk under reducing (left-hand side) or nonreducing (right-hand side, part shown only corresponding to area indicated on left-hand figure) conditions. The major milk proteins are indicated with arrows under reducing conditions; polymers from **Tables 1** and **2** are labeled with arrows only under nonreducing conditions.

Table 1. Characteristics of Protein and Polymer Spots Separated by Two-Dimensional Electrophoresis in Raw Milk under Reducing (*) or Nonreducing (**) Conditions from Image Analysis of Gels Presented in **Figure 2**[a]

	theor/app MW (kDa)	theor/app p*I*	no. of spot isoforms
α_{S1}-casein	22.97/33.6	4.91/4.66	1*
α_{S2}-casein	24.35/33.0	8.34/5.15−5.33	3*
β-casein	23.58/30.3	5.13/4.96−5.05	2*
κ-casein	18.97/24.4−26.1	5.93/4.64−6.24	10*
β-lactoglobulin	18.28/18.7	4.83/4.9−5.01	2*
α-lactalbumin	14.19/14.8	4.80/4.99	1*
serum albumin	66.43/65.4	5.6/5.88−6.25	6*
P1	Ø/79.1	Ø/4.95−5.29	7**
P2	Ø/75.9	Ø/5.02−5.23	6**
P3	Ø/64.1	Ø/4.94−5.76	9**
P4	Ø/69.2	Ø/4.94−5.76	9**
P5	Ø/64.6	Ø/4.69−4.82	5**
P6	Ø/71.9	Ø/4.62−4.78	5**
P7	Ø/86.2	Ø/4.74−5.85	18**
P8	Ø/75.9	Ø/5.81−5.92	3**

[a] Ø indicates that theoretical molecular weight (MW) or isoelectric point (p*I*) was not calculated for polymers P1 to P8.

isoforms arising from posttranslational modifications such as phosphorylation and glycosylation (*16*−*19*, *45*).

Under nonreducing conditions (**Figure 2**, right-hand side), several new spots appeared (P1 to P8), ranging from p*I* 4.62 to 6.25 and 64.1 to 86.2 kDa (**Table 1**). These polymers, naturally present in unheated milk, were only observed under nonreducing conditions (*45*, *46*) and were likely formed by disulfide cross-links between milk proteins. All of these species were analyzed by an ion-trap mass spectrometer coupled to a nanochromatography system, and the proteins identified as being involved in these complexes are reported in **Table 2**. All of the observed polymers were composed of α_{S2}-casein and/or κ-casein (P1 to P4), with α_{S1}-casein (P5, P6, and P7), β-casein (P8), β-lactoglobulin (P7), and serum albumin (P8). These polymers were observed with several spot isoforms (**Table 1**), from 3 spots (P8) to 18 spots (P7). The spot patterns were likely due to the biochemical characteristic of the proteins involved. For example, in the case of the homopolymer "P3", composed exclusively of κ-casein (*46*), 9 spots were

Table 2. Presence of Caseins and Whey Proteins in Polymers Observed in Raw Milk under Nonreducing 2DE according to Ref *45* and This Study

	α_{S1}-casein	α_{S2}-casein	β-casein	κ-casein	β-lactoglobulin	serum albumin
P1		x				
P2		x				
P3				x		
P4		x		x		
P5	x	x		x		
P6	x	x		x		
P7	x	x		x	x	
P8		x	x			x

observed and corresponded to the different posttranslational modifications of the protein. These results were in agreement with the data of Patel et al. (*43*), using a similar strategy for separation (PAGE instead of IEF for the first dimension), in which disulfide-bonded polymers of whey proteins, κ-casein, and α_{S2}-casein were identified after severe heat treatment (70−140 °C) of milk samples.

Quantification of Milk Proteins in Raw and Heated Milk. The total amount of each major milk protein separated under nonreducing 2DE was estimated for raw milk and milk heated for 5, 10, or 30 min at 90 °C (**Figure 3**). The quantification of these milk proteins was performed using the 2DE proteomic map of raw milk separated under reducing conditions as reference sample; each value corresponded to a percentage of the same protein on the reducing 2DE.

In raw milk (T0), 75% of β-lactoglobulin, 82% of α_{S2}-casein, and 54% of κ-casein spot volumes were present on nonreducing gels compared to gels run under reducing conditions. This decrease of spot amount implies that a significant proportion of these proteins are present in polymers in a native environment without any heat treatment. In contrast, serum albumin, α_{S1}-casein, and β-casein spots in heated milk were not significantly different under reducing and nonreducing conditions. After heating milk at 90 °C, serum albumin was the protein most affected, as around 85% of the protein disappeared on nonreducing 2DE gels after 5 min at 90 °C. Levels of β-lactoglobulin and κ-casein decreased gradually when milk samples were heated for 5, 10, and 30 min, until about 25% of protein remained. Under

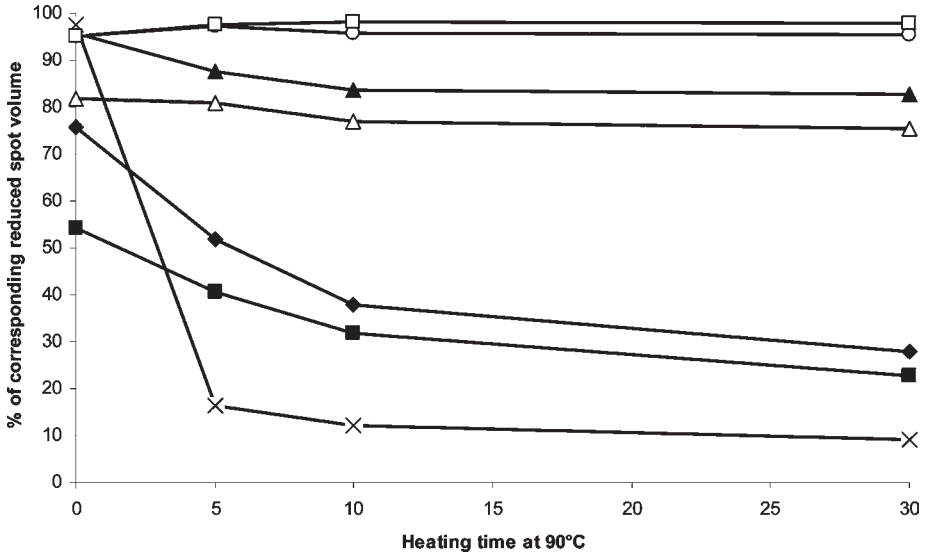

Figure 3. Levels of β-lactoglobulin (◆), κ-casein (■), α-lactalbumin (▲), serum albumin (×), α_{S1}-casein (○), α_{S2}-casein (△), and β-casein (□) separated under nonreducing 2DE normalized as a percentage of the corresponding spots separated under reducing 2DE as a function of heating time of milk at 90 °C.

nonreducing conditions, levels of α_{S1}-, α_{S2}-, and β-caseins were similar in unheated and heated samples. In contrast, the level of α-lactalbumin decreased from 95% (in raw milk) to 82% (heated for 30 min at 90 °C) under nonreducing conditions.

Quantification of Nonreduced Polymers in Raw and Heated Milk. As with the major milk proteins, all polymers observed under nonreducing conditions were quantified, and the proportion of each polymer under nonreducing 2DE was estimated (**Figure 4**, top part). In raw milk, the two most abundant polymers, P1 and P2, were composed of α_{S2}-casein only. Relative to the total of all polymer spot volumes, polymers involving only α_{S2}-casein represented 43% of all polymers, demonstrating the significance of intermolecular disulfide bonding for this protein. Polymers P2 to P7, which showed a larger p*I* range, were composed of homopolymers of κ-casein (12%), as observed by Holland et al. (*46*) and/or a combination of α_{S1}-casein, α_{S2}-casein, and β-lactoglobulin. Serum albumin and β-casein were only identified in polymer P8, which represented only 2% of the amount of all polymers. It can be noticed that α_{S2}-casein and κ-casein were involved in 45% and 31% of polymers, respectively, as heteropolymers. This emphasizes the key role of the cysteine residues of these two proteins in forming naturally occurring interchain disulfide bridges between proteins in raw milk (*22*, *49*, *50*).

In heated milk, changes in the level of each polymer using the level of all polymers observed in raw milk as reference was also analyzed (**Figure 3** bottom part). Polymers P1, P3, P6, and P7 remained constant and polymers P2, P4, P5, and P8 were significantly decreased as a function of heating time. Polymer P8 was the most affected by heat treatment, with a reduction of 80% of the polymer level being observed after 5 min at 90 °C.

As polymers P1 and P2 were composed of homopolymers of α_{S2}-casein and level of P1 remained constant, but that of P2 decreased, as a function of heating time, it is proposed that polymer P2 may be a stable transition complex, which can be converted to P1 during heat treatment.

Quantification of Individual κ-Casein Spots in Raw and Heated Milk. In raw milk, 10 κ-casein spots were observed under reducing conditions (**Figures 2** and **5A**), using an IEF range p*I* 4−7. This spot pattern was consistent with the study of Holland et al. (*16*), who analyzed the posttranslational modifications of κ-casein in bovine milk, using 2DE (p*I* 4−7 and 14% acrylamide SDS−PAGE).

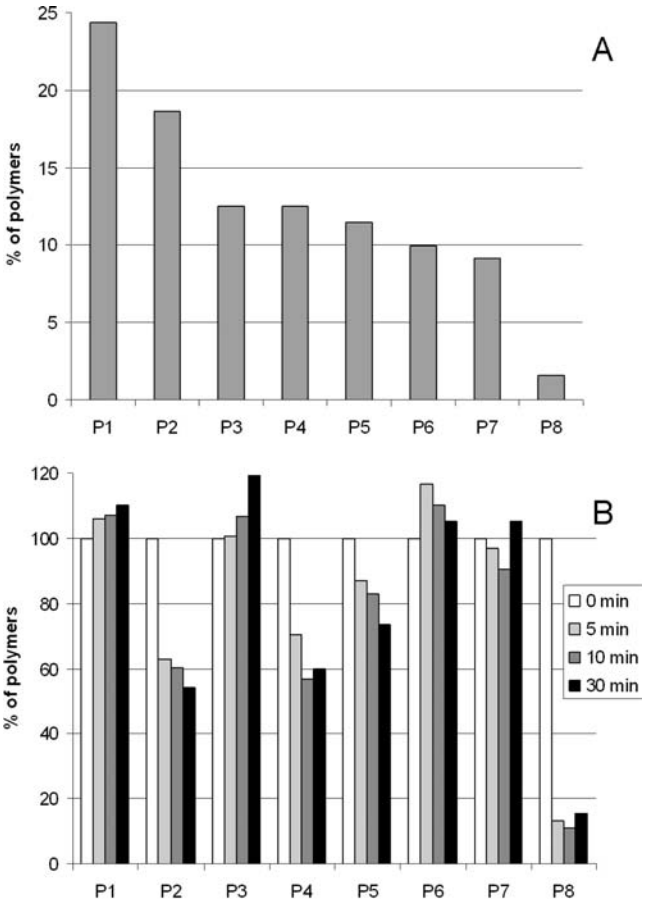

Figure 4. Proportion (%) of each polymer among all polymers observed in raw milk under nonreducing 2DE (**A**) and as a function of heating time of milk at 90 °C (0, 5, 10, and 30 min) using the corresponding spots of raw milk as reference (**B**).

Under nonreducing 2DE conditions, most of the spot volumes of κ-casein decreased (**Figure 5B**) as compared with reducing conditions, but not in the same proportions.

The 10 spots observed under reducing conditions were used as a reference to analyze spot volume under nonreducing conditions

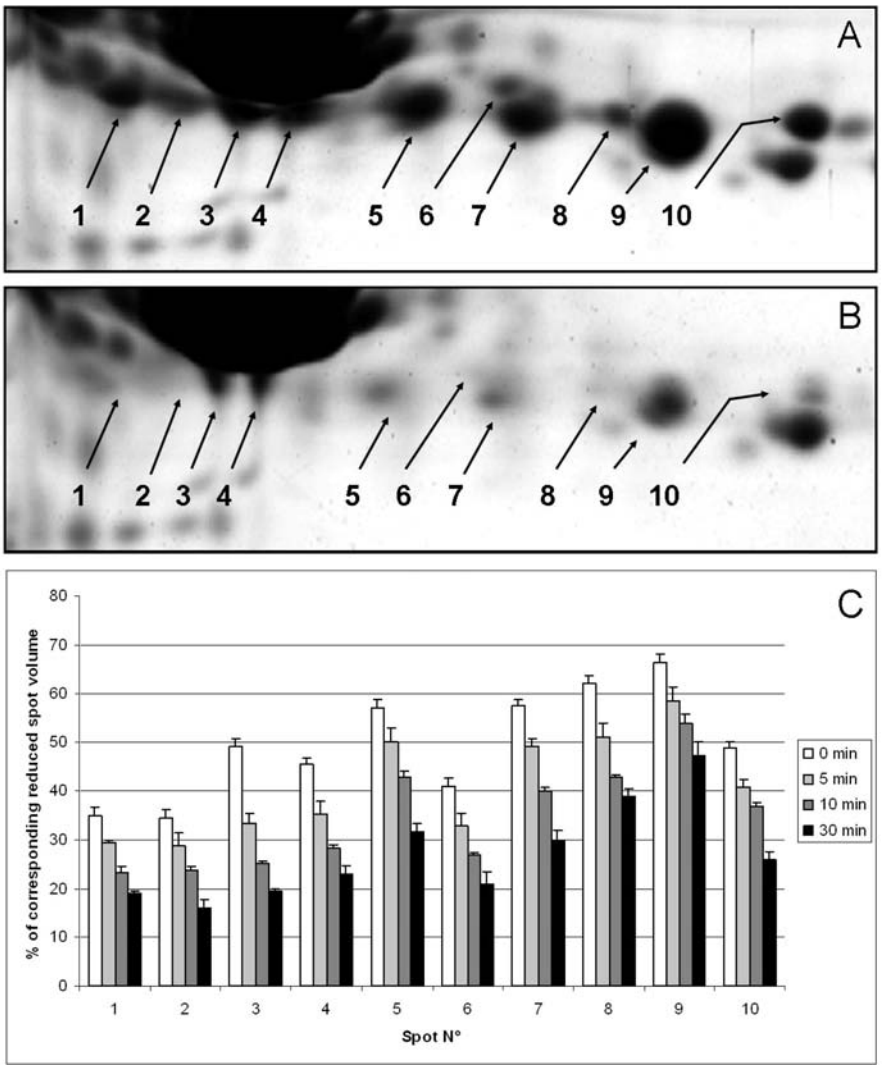

Figure 5. Quantification of individual κ-casein spots. The 10 observed κ-casein spots were labeled on the 2DE map of raw milk proteins separated under reducing conditions (**A**) or under nonreducing conditions (**B**). Each spot, separated under nonreducing conditions, was quantified and displayed as a percentage of the corresponding spots separated under reducing 2DE as a function of heating time of milk at 90 °C (**C**).

in raw milk (T0) or following heat treatment of milk at 90 °C (**Figure 5C**). In raw milk, the two spots with the greatest decrease under nonreducing condition were the most acidic κ-casein spots, and the least affected spots were the most basic κ-casein spots (except spot 10). During heat treatment at 90 °C, all spot volumes decreased, but the same proportions between acidic and basic κ-casein spots remained.

This quantitative analysis of κ-casein spots showed that sensitivity of the isoforms to heat treatment was potentially related to posttranslational modifications of the proteins, which is related to protein localization and orientation inside the casein micelle (*21*−*23*, *51*).

DISCUSSION

The analysis of complexes formed during heat treatment of bovine milk is very difficult due to the inherent complexity of the milk system. In particular, the caseins are organized as casein micelles in which proteins are held together by a combination of hydrophobic associations and covalent cross-links (*20*, *23*, *52*). To study all protein interactions in their natural form, or after heat treatment, an original strategy was developed which involved nonreducing and reducing combination steps during the two-dimensional gel electrophoresis protocol (*45*). Under nonreducing conditions, milk protein solubilization and all of the steps of the 2DE separation were performed without DTT. Prior to the second dimension, strips were incubated in equilibration buffer containing urea and SDS, followed by an equilibration step containing iodoacetamide. In the case of the reduced samples, solubilization of milk proteins was achieved with DTT as well as the first equilibration step. To obtain a good separation of polymers on the top of the 2D gel, homemade gradient polyacrylamide gels (10−18%) were used during the second dimension. After image analysis, and identification of proteins and polymers by mass spectrometry, a detailed and quantitative description of native interactions between milk proteins can be performed, and possible roles of each major milk protein can be described.

Presence of κ-Casein Polymers under Native Conditions. Bovine κ-casein consists of multiple isoforms due to several genetic variants and differential phosphorylation and glycosylation. This casein also contains two cysteine residues, allowing the formation of an intramolecular disulfide bond (*50*) or the assembly of disulfide-linked polymers (*53*−*55*). Isoforms of κ-casein can be separated by 2DE in the p*I* range 4−7 as 10 major spots (*16*, *45*). Spots corresponding to genetic variant, or forms differing in phosphorylation and/or glycosylation, have been identified and the posttranslational modifications located, using a mass spectrometry approach (*17*, *19*). In the present study, using a global 2DE approach to separate all major milk proteins on the same gel,

10 spots of monomeric κ-casein were observed under reducing conditions (**Figures 2** and **5**) ranging from p*I* 4.6 to p*I* 6.2. Under nonreducing conditions, the intensity of most spots was decreased, showing the presence of polymers. Image analysis focused on monomeric κ-casein revealed that the most acidic spots were highly involved in polymer formation (**Figure 5C**). These spots corresponded to the proteins with a complex pattern of posttranslational modifications. The most acidic spots observed on a 2DE gel of milk are reported to have three glycosylations and one phosphorylation site (*16*). According to the spot volume quantification, it seemed that such posttranslational modifications increased oligomerization of κ-casein. Phosphorylation and glycosylation modified the charge of κ-casein, as observed on a 2DE gel by a shift in isoelectric points.

As previously described for the tau protein with self-assembly modification into paired helical filaments (*56*), phosphorylation could also generate structural modifications of κ-casein inside the casein micelle, inducing a potential higher reactivity of cysteine residues to form interchain disulfide polymers. Oligopolymers of κ-casein were observed on 2DE gels of bovine milk under nonreducing conditions (*46*). Trains of spots corresponding to monomers to hexamers were observed as a result of the participation of different glycoforms and phosphoforms in oligomer formation. In this study, the polymers were observed at the same molecular weight range as in ref *46*, but sometimes with a more complex protein composition (**Figure 2** and **Table 2**).

This strategy of analysis not only focused on identification of κ-casein polymers but also allowed the complete study of all species involved in polymer formation. In agreement with ref *46*, κ-casein was observed in polymers P3, P4, and P7, but α_{S2}-casein was also identified in polymer P4, and a mixture of α_{S2}-casein, α_{S1}-casein, and β-lactoglobulin was identified in polymer P7. No polymer of α_{S2}-casein and κ-casein has been described previously. Disulfide-linked homopolymers of α_{S2}-casein and homopolymers of κ-casein have been observed under various environments and treatments (*50*, *57*). As no disulfide linkage between proteins was analyzed and identified by mass spectrometry, it is not possible to distinguish between heteropolymers and a mixture of homopolymers inside the same spot. Nevertheless, only single and well-separated spots were excised and analyzed by mass spectrometry (**Figure 2**). As all analyzed and quantified polymers displayed a characteristic pattern (**Table 1**), it is unlikely that two different homopolymers can be identified inside the same spot, even if this cannot be totally excluded. In addition, α_{S2}-casein has been shown to display an increased accessibility and ability to form complexes involving κ-casein and whey proteins after high-pressure treatment (*43*, *58*). Thus, there is substantial evidence for the capacity of κ-casein and α_{S2}-casein to form disulfide-linked heteropolymers.

Involvement of α_{S2}-Casein in Native Milk Polymers. Of the four caseins, only α_{S2}-casein and κ-casein contain cysteines. Of these, α_{S2}-casein naturally occurs as monomers and dimers in bovine milk (*59*). In the present study, the two major polymers observed under nonreducing 2DE corresponded to homopolymers of α_{S2}-casein, representing 43% of all polymers observed in raw milk. Since α_{S2}-casein contains two cysteine residues (amino acids 36 and 40), a dimer could be linked by one or two disulfide bridges. If two monomers are linked by one intermolecular disulfide bridge, free cysteine residues remain and can react with other proteins to form several kind of polymers. In this study, several proteins in raw milk containing cysteine residues, i.e., κ-casein, β-lactoglobulin, and serum albumin (**Table 2**), were identified inside complexes involving α_{S2}-casein. Using a similar approach, Bouguyon et al. (*52*) observed several homopolymers and heteropolymers involving rat or mouse α_S-casein, β-casein, and κ-casein linked by disulfide bridges. As observed in **Figure 3**, only a low percentage of monomeric α_{S2}-casein was involved in polymers, but this amount was enough to promote disulfide-linked polymers under native conditions. Interactions between casein in micelles are governed mainly by a balance of attractive hydrophobic interactions and electrostatic repulsion (*21*), but disulfide bridges between caseins are now considered to help in contributing to stabilization of the casein micelle (*22*, *23*).

Interaction of Whey Proteins with the Casein Micelle during Heat Treatment. κ-Casein is located mainly at the micelle surface, where it plays a role in regulating micelle size and in maintaining them in suspension in milk (*21*). The peripheral location of κ-casein in the micelle stabilizes the protein and acts as a bridge to link proteins of the whey fraction and caseins hidden inside the micelle. In this study, β-lactoglobulin was identified in polymer P7 in raw milk samples, mixed with κ-casein, α_{S1}-casein, and α_{S2}-casein. Serum albumin was also identified in polymer P8, mixed with α_{S2}-casein and β-casein (**Table 2**). As α_{S2}-casein and κ-casein are the only caseins which contain cysteines, α_{S1}-casein and β-casein were not linked to the other proteins by disulfides bridges but possibly by other bonds such as glycation cross-links (*60*) or strong ionic/hydrophobic interactions, not broken by SDS during electrophoresis. Levels of monomeric β-lactoglobulin and serum albumin decreased rapidly with heating time (**Figures 1** and **3**), probably through the formation of polymers involving caseins and whey proteins (*32*−*34*, *61*−*63*). Polymer formation involving β-lactoglobulin and κ-casein has been studied extensively for 40 years under a huge variety of environments and treatments (*38*, *64*−*68*). However, according to the present study, only a few detectable polymers contained whey proteins (P7 and P8), and these represented only 11% of the polymers observed on the 2D gels of raw milk samples under nonreducing conditions (**Figure 4**). Using a nonreducing/reducing discontinuous strategy (nonreduced isoelectric focusing and then reduced SDS−PAGE), we previously showed the presence of large polymers composed of serum albumin, κ-casein, and β-lactoglobulin in milk heated at 90 °C (*45*). Using such a strategy, proteins were visualized on the 2D gel as polymers were reduced to monomers. In the present study, polymers were only observed under nonreducing conditions, and larger polymers/aggregates, which were not able to enter the second dimension gel, cannot be visualized and quantified. A gradient gel (10−18% acrylamide) was used as second dimension to reduce this problem, but this limitation still remains for polymers greater than 200 kDa.

Interactions of Caseins To Prevent Formation of Fibrils: A Possible Biological Mechanism. Under nonreducing conditions, α_{S2}-casein and κ-casein were associated in milk as homodimers and/or heterodimers or multimers (this study and refs *22*, *46*, *49*, *50*, *53*, *54*, and *57*). Under physiological conditions, caseins have little secondary or tertiary structure with extensive regions of disordered structure (*20*−*22*, *51*). These proteins are organized and stabilized with calcium inside micelles, but under stress conditions such as heat treatment or a reducing environment, associations between caseins can be disrupted, and proteins can adopt amyloid fibril structures associated with degenerative diseases (*26*−*28*, *69*−*72*). Native κ-casein is organized as a series of disulfide-bonded polymers (*46*), ranging from dimers to octamers and above. When cysteine residues are chemically modified to prevent disulfide bond formation (by reduction and carboxymethylation), κ-casein increases in molecular weight and shows a tendency toward self-association, in particular the formation of fibrillar structures (*70*).

Of the caseins, α_{S2}-casein is the least abundant and perhaps the most difficult to isolate in a pure form. Consequently, it has been the least studied. After reduction of disulfide bridges, α_{S2}-casein is particularly susceptible to fibril formation under physiological

conditions (*27*). Consequently, self-associations and interactions between caseins inside the casein micelle can prevent such aberrant fibrillar structures.

In conclusion, α_{S2}-casein and κ-casein in raw milk were shown to participate in a complex pattern of homopolymers and heteropolymers in association with the other caseins and whey proteins under native-like nonreducing conditions, and α_{S2}-casein was the milk protein most implicated in polymer establishment and heterogeneity. Only a small proportion of α_{S2}-casein was present in polymers, but this corresponded to a large proportion of all polymers identified. This protein appeared as a platform which was able to connect several milk proteins using disulfide bonds and probably other kinds of links with cysteine-free proteins. The technical strategy using two-dimensional electrophoresis under reducing and nonreducing conditions was very useful in revealing the partners involved in polymers, but it also showed the limit of gel-based electrophoresis, which was not effective in analysis of large complexes. Posttranslational modification of κ-casein increased its sensitivity to oligomerization, but further study is required to further define the role of the protein in polymer formation. All major milk proteins appeared to interact, suggesting a very intricate system inside casein micelles and between caseins and whey proteins. Prevention against aberrant fibril structures of caseins is proposed to explain at least in part milk protein interactions and polymer stability.

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