JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Heat and High Hydrostatic Pressure Treatments on Disulfide Bonding Interchanges among the Proteins in Skim Milk

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Traditionally, milk has been heat treated to control microorganisms and to alter its functionality, for example, to increase its heat stability. Pressure treatment has been considered as a possible alternative for microorganism control, but some of the functionality-related milk protein interactions have not been explored. The present study used two novel two-dimensional polyacrylamide gel electrophoresis (2D PAGE) methods to explore the differences in the irreversible disulfide bond changes among the milk proteins after four common heat treatments and after 30-min pressure treatments of milk at 200, 400, 600, and 800 MPa at ambient temperature (22 °C). The pasteurizing heat treatment (72 °C for 15 s) denatured and aggregated only a few minor whey proteins, but the high heat treatments (100 °C for 120 s, 120 °C for 120 s, and 140 °C for 5 s) formed disulfide-bonded aggregates that included a high proportion of all of the whey proteins and κ-casein (κ-CN) and a proportion of the α_{s2}-CN. Pressure treatment of milk at 200 MPa caused β-lactoglobulin (β-LG) to form disulfide-bonded dimers and incorporated β-LG into aggregates, probably disulfide-bonded to κ-CN. The other whey proteins appeared to be less affected at 200 MPa for 30 min. In contrast, pressure treatment at 800 MPa incorporated β-LG and most of the minor whey proteins, as well as κ-CN and much of the α_{s2}-CN, into aggregates. The accessibility of α_{s2}-CN and formation of complexes involving α_{s2}-CN, κ-CN, and whey proteins in the pressure treated milk is an important novel finding. However, only some of the α-lactalbumin was denatured or incorporated into the large aggregates. These and other results show that the differences between the stabilities of the proteins and the accessibilities of the disulfide bonds of the proteins at high temperature or pressure affect the formation pathways that give the differences among the resultant aggregates, the sizes of the aggregates, and the product functionalities.

KEYWORDS: High pressure; high temperature; protein aggregation; two-dimensional PAGE; disulfide bonds; β-lactoglobulin; κ-casein; α_{s2}-casein; whey proteins; casein micelle

INTRODUCTION

Thermal treatment of milk and milk products is an essential operation in commercial dairy processes in order to provide acceptable safety and shelf life or to improve the functional properties of final products (*1*, *2*). The heat-induced interactions of milk proteins play an important role in the heat-induced functionality of many dairy products. Examples of -heat-induced functionality include improvement in the heat stability of milk, cheese-making properties, and the texture of yogurt.

Heat treatment of milk at >70 °C during commercial processing operations 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 the κ-casein (κ-CN) of the casein micelles (*2*−*9*), that subsequently influence the functional properties of milk products. Many studies have reported interactions between β-lactoglobulin (β-LG) and κ-CN in model systems or in milk. It has been reported in these studies that two disulfide bridges and a free sulfhydryl group present in the native structure of β-LG seem to play an important role in its heat-induced interactions with κ-CN (*2*, *6*−*11*). However, the severe heat treatments often have adverse effects on the color, flavor, texture, and nutritional quality of the final products. In many cases, high hydrostatic pressure (HP) treatment in the range of 100−1000 MPa has been suggested as an alternative nonthermal treatment for pasteurization or sterilization, with

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10.1021/jf052834c CCC: $33.50
Published on Web 04/08/2006

minimal effects on the sensory and nutritional quality of foods (*12*−*14*). It has also been recognized as a tool for the modification of macromolecular constituents, such as proteins (*12*, *13*, *15*), and thus is considered to be one of the most promising new methods for modifying the functional and textural properties of dairy food products (*16*−*21*).

Studies have shown that HP denatures proteins (*13*) and may bring about reversible and irreversible changes in the conformation of proteins (*22*). Much of the work on the effects of HP on milk proteins has been done on the denaturation behavior (or kinetics) of individual proteins in raw milk (*23*−*28*), pasteurized milk (*20*, *21*), reconstituted skim milk (*29*), and whey protein concentrate (WPC) solutions (*30*, *31*). These studies reported that the pressure stabilities of β-LG, α-lactalbumin (α-LA), bovine serum albumin (BSA), and immunoglobulins (Igs) were different and that β-LG was the most sensitive and α-LA the most resistant to pressure treatment. β-LG is reported to interact with κ-CN (*32*), probably via disulfide bonds (*21*, *25*, *26*, *33*, *34*). Some of these studies reported a decrease in the turbidity and the size of the casein micelles due to their disintegration as a consequence of pressure treatments between 250 and 600 MPa (*21*, *28*, *29*, *35*, *36*). Most of these studies explored the effects of HP on individual proteins, but little attention has been paid to the interactions of caseins and whey proteins in model systems or in small-scale processing plants.

Over the past decade, we have developed various one-dimensional (1D) and two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) techniques to characterize aggregates and interaction products formed in heat-treated solutions of β-LG (*37*), heat-treated WPC solutions (*38*), or HP-treated WPC solutions (*31*). These studies suggested detailed pathways involving heat-induced aggregation and gelation of whey proteins, including the roles of different types of proteins and forces (disulfide bonding and hydrophobic interactions) involved in the formation of the heat-induced aggregates of whey proteins.

We have recently studied the effects of high pressure on WPC solutions (*30*, *31*) and found that β-LG was the most important protein for pressure-induced gel formation and that there was a significant difference between the consequences of pressure treatments of 200 and 600 MPa. The relative stabilities of β-LG and α-LA were similar in the heated systems (*38*), and this was a consequence of the ability of α-LA and β-LG to form some kind of entity that allowed disulfide bond interchange between α-LA and β-LG. However, this did not occur via the same mechanism in the pressure-treated WPC solutions.

In the present study, four commercial heat treatments were applied to raw skim milk using a small-scale pilot plant. For comparison, milk from the same source was pressurized at 200, 400, 600, and 800 MPa at 22 °C for 30 min. It was necessary to develop two further novel PAGE methods to analyze the large process-induced aggregates in the presence of high casein concentrations.

MATERIALS AND METHODS

A summary of the experimental plan for generating and analyzing the heat- and pressure-treated skim milk samples by PAGE methods is outlined in **Figure 1**.

Materials. The electrophoresis chemicals were obtained from Bio-Rad Laboratories (Hercules, CA). Reducing agent (2-mercaptoethanol, 2-ME) was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade from BDH Laboratory Supplies (Poole, U.K.). Artesian bore water was purified by reverse osmosis treatment followed by carbon treatment and deionized using a Milli-Q apparatus (Millipore Corp., Bedford, MA).

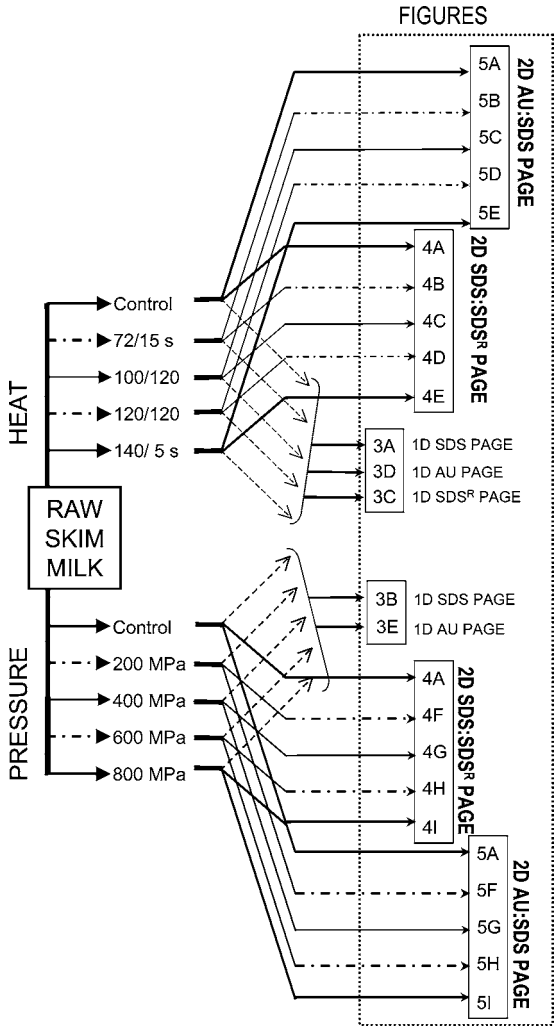

Figure 1. Outline of the steps taken from the raw skim milk, via a variety of heat and pressure treatments, to the PAGE analysis results. The heat treatments used were comparable with those used in industry and corresponded to pasteurization (72 °C for 15 s), functional modification (100 °C for 120 s or 120 °C for 120 s), and sterilization (140 °C for 5 s). The pressure treatments for 30 min had no relevance to any particular industrial process. PAGE results are shown in the identified figures.

About 40 L of fresh bulk cow's milk (from a morning milking) was obtained for each trial from a dairy farm at Massey University, Palmerston North, New Zealand. The milk was warmed to 40 ± 1 °C and skimmed (without pasteurization) using a pilot-scale cream separator. The fresh skim milk, which had a fat content of <0.1% w/w, was used in subsequent experiments.

Heat Treatments of Skim Milk. Four aliquots of skim milk (4 L each) were heated at 72 °C for 15 s, at 100 °C for 120 s, at 120 °C for 120 s, or at 140 °C for 5 s in a pilot-scale UHT plant (Type D, α-Laval, Sweden) installed in the Massey University pilot plant. The time−temperature combinations used in this experiment represent four of the routinely used commercial heat treatments in the New Zealand dairy industry [viz., pasteurization, two preheat treatments used in the powder manufacturing process, and ultrahigh temperature (UHT) sterilization, respectively]. The milk was heated in a spiral tube heat-exchanger, with the required holding time adjusted by altering the flow of the incoming milk, and by selecting appropriate holding tubes. The milk was subsequently cooled by a combination of flash evaporation and heat exchange.

High-Pressure Treatments of Skim Milk. Aliquots of skim milk were transferred into Beckman Polyallomer Quick-Seal (13 mm i.d., 51 mm high) centrifuge tubes (Beckman Instruments, Inc., Spinco Division, Palo Alto, CA) at 20 °C. The tubes were then heat sealed and transferred into the pressure chamber of a Stansted Fluid Power

"Food-Lab" high-pressure unit maintained at 22 °C. The dimensions of the chamber of the high-pressure rig were 17 mm × 132 mm. The pressurization and depressurization rates were 5 and 14.5 MPa/s, respectively. The average adiabatic heating during pressurization was 1.4 °C/100 MPa, and the cooling during depressurization was 1.3 °C/100 MPa (*36*). A homogenized emulsion of vegetable oil (10%) in water containing surfactant and preservative was used as the pressurizing fluid. The temperature of the pressure-transmitting fluid returned to 22 °C in <5 min (*36*) after pressurization or depressurization. All of the samples in the present study were analyzed after pressure release, and no sample was analyzed under pressure.

Sample Preparation and PAGE Analysis. Subsamples of the control (untreated) and heat- or pressure-treated milk samples were subsequently diluted in a sodium dodecyl sulfate (SDS) sample buffer that contained 5 M urea. An aliquot of each of these diluted subsamples was mixed with a stacking gel mixture and immediately placed in the appropriate loading well of the SDS-PAGE gels and allowed to set.

A further set of each subsample (1 mL) was mixed with 20 μL of 2-ME and heated at 94 °C for 4 min to reduce the disulfide bonds. These subsamples were then mixed with SDS sample buffer and loaded onto SDS gels together with a set of molecular weight standards.

A third set of subsamples was diluted into alkaline urea sample buffer and then analyzed by urea-PAGE.

The preparation and running of the gels, and their staining and destaining, photography, and quantitative scanning, followed the procedures described by Havea et al. (*38*) and Manderson et al. (*37*) for the SDS and SDS of reduced samples (SDS^R) techniques, the procedure described by Creamer (*39*) for the alkaline urea (AU) technique, and the procedure described by Davis (*40*) for the "set sample" technique.

The 2D SDS:SDS^R-PAGE method described by Havea et al. (*38*) was used with some modifications to analyze pressure-treated, heat-treated, and control samples. The major difference between the method reported in this paper and that reported earlier (*38*) was that the sample strip contained the set sample gel (**Figure 2**). The sample was mixed with the stacking gel mixture and was set within the sample loading well. This modification was required to trap very large aggregates (the aggregates that were unable to enter the gel) (**Figure 2**).

The 2D AU:SDS-PAGE method has not been reported previously for milk. In this method, the excised sample strip from first dimension urea-PAGE containing 5 M urea was immersed in SDS sample buffer for 4 min, briefly washed with water, and placed between the two glass plates prior to having the resolving and stacking gels set around the sample gel strip, as described by Havea et al. (*38*).

RESULTS

The standard PAGE methods of alkaline- (or native-) and SDS-PAGE were not suitable for dispersing the proteins sufficiently to give clear patterns of the various proteins. The caseins, which are naturally aggregated into micelles, are not readily dispersed at neutral pH. However, in SDS solutions, the component caseins are readily dispersed as SDS complexes. After reduction of the disulfide bonds in κ-CN and α_{s2}-CN, all four caseins (α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN) are readily separated by SDS^R-PAGE, but they have similar mobilities and the bands overlap when the samples contain too much casein. It was found that the caseins are also dispersible in 4.5 M urea solution and migrate satisfactorily at higher sample loadings in a urea-PAGE system. A preliminary study showed that a 2D PAGE system based on a urea separation in the first dimension followed by an SDS separation could effectively separate the caseins from one another.

A second technical problem was that some very large heat- or pressure-induced aggregates (molecular mass > 500 kDa) did not enter the stacking gel during electrophoresis; as a consequence, they were caught in the sample loading well and were subsequently lost (washed away) during the staining and destaining procedures. Again, a preliminary study showed that

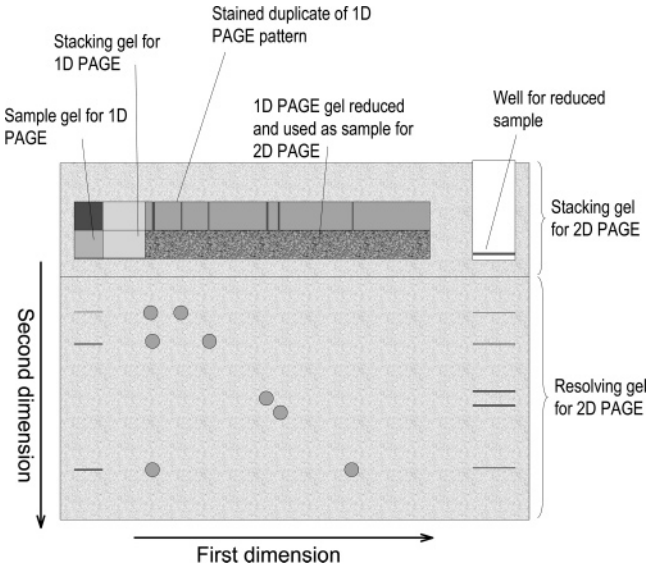

Figure 2. Diagram showing the preparation and running of a sample of heated milk in a 2D SDS:SDS^R-PAGE system. The milk sample is dispersed in SDS buffer, mixed with an acrylamide gel solution, and set in two sample wells of a standard SDS-PAGE gel. After electrophoresis of the samples, one of the gel columns is stained and the other is immersed in a dilute 2-ME solution for 4 min at 94 °C to reduce the covalent disulfide bonds. After washing, the unstained treated gel is clamped into another electrophoresis cell, the resolving gel is set under it, and then the stacking gel is set around it. All of the buffers contain SDS. After the second electrophoresis, staining, and destaining, the previously stained gel column is placed above and parallel to the column that had been set into the gel. This setup is then scanned and photographed (see **Figure 4** for examples). This procedure allows the relationship between the stained spots/bands of the disulfide-bonded proteins and the reduced proteins to be established.

setting the original control or heat- or pressure-treated samples with a polyacrylamide gel of composition similar to that of the stacking gel and setting them in the sample loading well as described by Davis (*40*) trapped the protein aggregates with a molecular mass > ~500 kDa within set sample gels. This strategy prevented the loss of very large protein aggregates during electrophoresis and the gel staining and destaining procedures. Consequently, an estimate of the qualitative composition of these aggregates could be made using the 2D SDS:SDS^R-PAGE procedure. These techniques were then used in this study.

1D PAGE. The preparation of the heat- and pressure-treated milk samples is outlined in **Figure 1**, and the resultant 1D SDS- and urea-PAGE patterns are shown in **Figure 3**. The major milk proteins were identified in the control samples (*41*) (lanes 1 and 6 in **Figure 3A**−**E**), and their identities are noted on the left-hand side of each gel pattern (**Figure 3A**,**C**−**E**). Panels **A** and **B** of **Figure 3** show the SDS-PAGE patterns and panels **D** and **E** the urea-PAGE patterns of the same samples as reported in **Figure 3A**,**B**.

Some large aggregates [marked X1 (**Figure 3A**,**B**,**D**,**E**) and Y′ and Y″ (**Figure 3D**,**E**)] were present in the control samples (lane 1 in each gel).

The heat- and pressure-induced large aggregates (lanes 2−5) were classified into three groups and noted as regions X2, X3, and X4. In **Figure 3A**,**B**, X4 was the material trapped in the set sample gels; in **Figure 3D**,**E**, X4 contained the material that would normally be caught in the sample loading well at the top of the stacking gel.

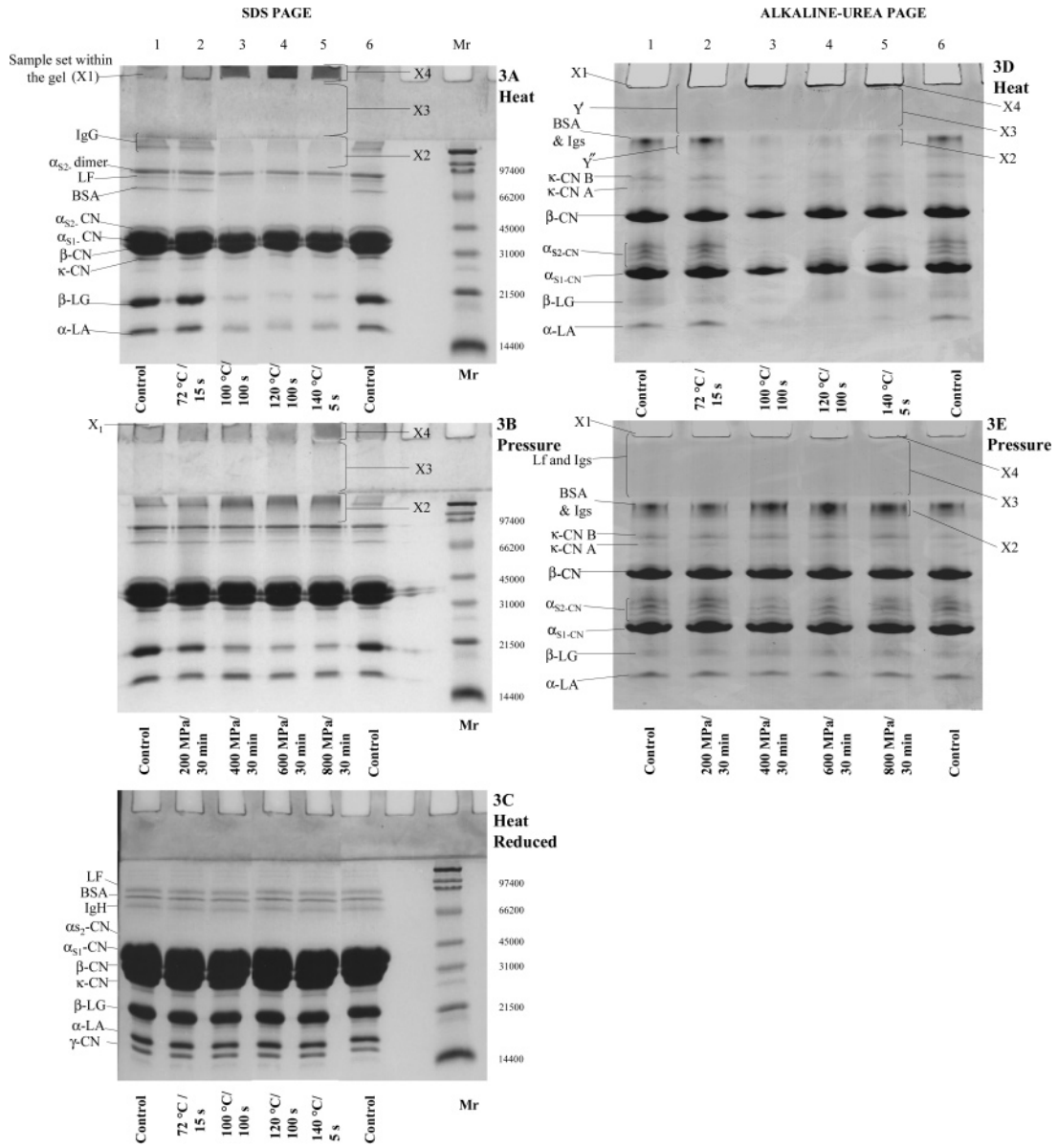

Figure 3. 1D SDS-PAGE patterns of nonreduced control, heat-treated (**A**), and pressure-treated (**B**) samples, and SDS-PAGE pattern of reduced heat-treated samples (**C**); similarly, 1D AU-PAGE patterns of heat-treated (**D**) and pressure-treated (**E**) samples. Sample loading details in the PAGE patterns of the heat-treated samples (**A**, **C**, and **D**) show control (lane 1) and heat-treated at 72 °C for 15 s (lane 2), 100 °C for 120 s (lane 3), 120 °C for 120 s (lane 4), 140 °C for 5 s (lane 5), and control (lane 6). Similarly, PAGE patterns of the pressure-treated samples (**B** and **E**) show control (lane 1) and samples pressure treated at 200 MPa for 30 min (lane 2), 400 MPa for 30 min (lane 3), 600 MPa for 30 min (lane 4), 800 MPa for 30 min (lane 5), and control (lane 6).

SDS-PAGE of Heat-Treated Samples. The heat-treated samples were analyzed by SDS-PAGE (**Figure 3A**, lanes 2−5) and, after reduction with 2-ME, by SDS^R-PAGE (**Figure 3C**, lanes 2−5). In the PAGE pattern of the reduced samples (**Figure 3C**), the samples in lanes 1−6 appeared to be similar, indicating that none of the polypeptide bonds was cleaved by the heat treatment and that no irreducible covalent bonds were formed.

By comparison, the unreduced samples (**Figure 3A**) gave significantly different patterns, as noted by the significantly lower intensity of the band corresponding to β-LG and the markedly lower intensity of the band corresponding to α-LA in the severely heated samples (lanes 3−5). However, it can be noted that the mild heat treatment (72 °C for 15 s, lane 2) did not affect the intensities of the bands corresponding to β-LG and α-LA. However, the other minor whey proteins [such as IgG, lactoferrin (LF), and BSA] were also very labile, and some reduction in the intensity of the bands corresponding to monomeric IgG, LF, and BSA was observed in the PAGE pattern of the mildly heat-treated samples (lane 2); the bands corresponding to these heat-sensitive proteins were essentially absent in the PAGE patterns of the severely heat-treated samples (lanes 3−5). The patterns in these lanes also showed significant density in the set sample gels (X4), indicating that a significant quantity of large protein aggregates was present in the milks heated at 100 °C for 120 s (lane 3), at 120 °C for 120 s (lane 4), and at 140 °C for 5 s (lane 5).

Urea-PAGE of Heat-Treated Samples. Analysis of the control milks using urea-PAGE (**Figure 3D**, lanes 1 and 6) showed that the caseins were well separated from one another. Also, the effects of various genetic polymorphs of proteins, post-translational modifications, phosphorylation, or glycosylation (*41*) could be seen in some of the samples. β-LG gave rather blurred bands, probably as a consequence of thiol−disulfide interchange in the alkaline (pH ≈8.7) chaotropic environment.

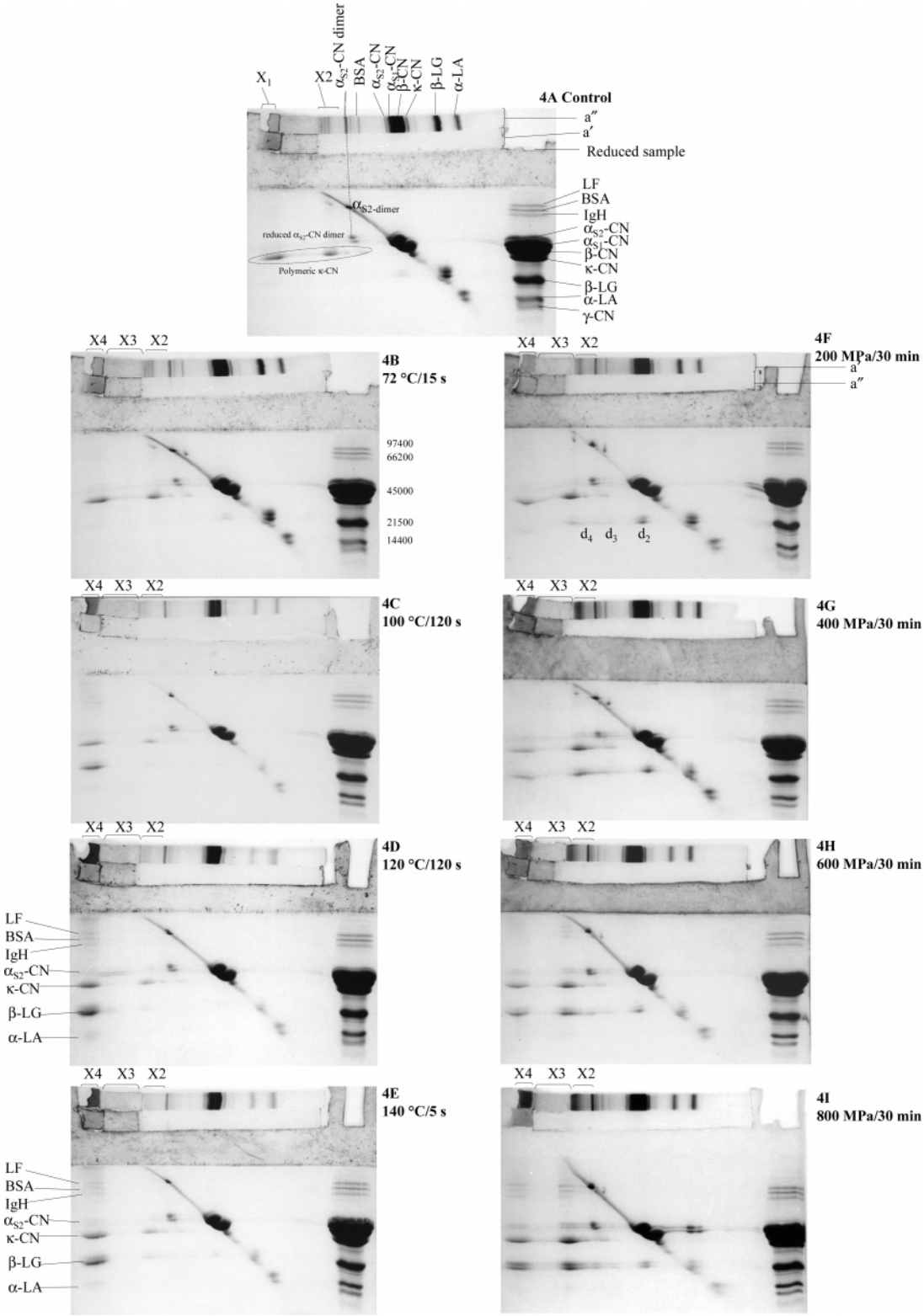

Figure 4. 2D SDS- then reduced SDS-PAGE patterns of control (**A**) and heat-treated samples [72 °C for 15 s (**B**); 100 °C for 120 s (**C**); 120 °C for 120 s (**D**); 140 °C for 5 s (**E**)]; similarly, 2D PAGE patterns of pressure-treated sampless [200 MPa for 30 min (**F**); 400 MPa for 30 min (**G**); 600 MPa for 30 min (**H**); 800 MPa for 30 min (**I**)].

Analysis of the heat-treated milks using urea-PAGE (**Figure 3D**, lanes 2−5) showed that the mild treatment (72 °C for 15 s) gave a pattern (**Figure 3D**, lane 2) that was almost indistinguishable from the controls (**Figure 3D**, lanes 1 and 6). The samples that had been heated more extensively (**Figure 3D**, lanes 3−5) contained very little whey protein or κ-CN and diminished quantities of α_{s2}-CN. Corresponding quantities of large aggregates were caught at the top of the stacking gel or were present in the sample loading well. These results are consistent with those shown in **Figure 3A**.

SDS-PAGE of Pressure-Treated Samples. Analysis of the pressure-treated samples using SDS-PAGE (**Figure 3B**) showed steady changes with the extent of high-pressure treatment from 200 to 800 MPa (lanes 2−5). β-LG and some of the minor

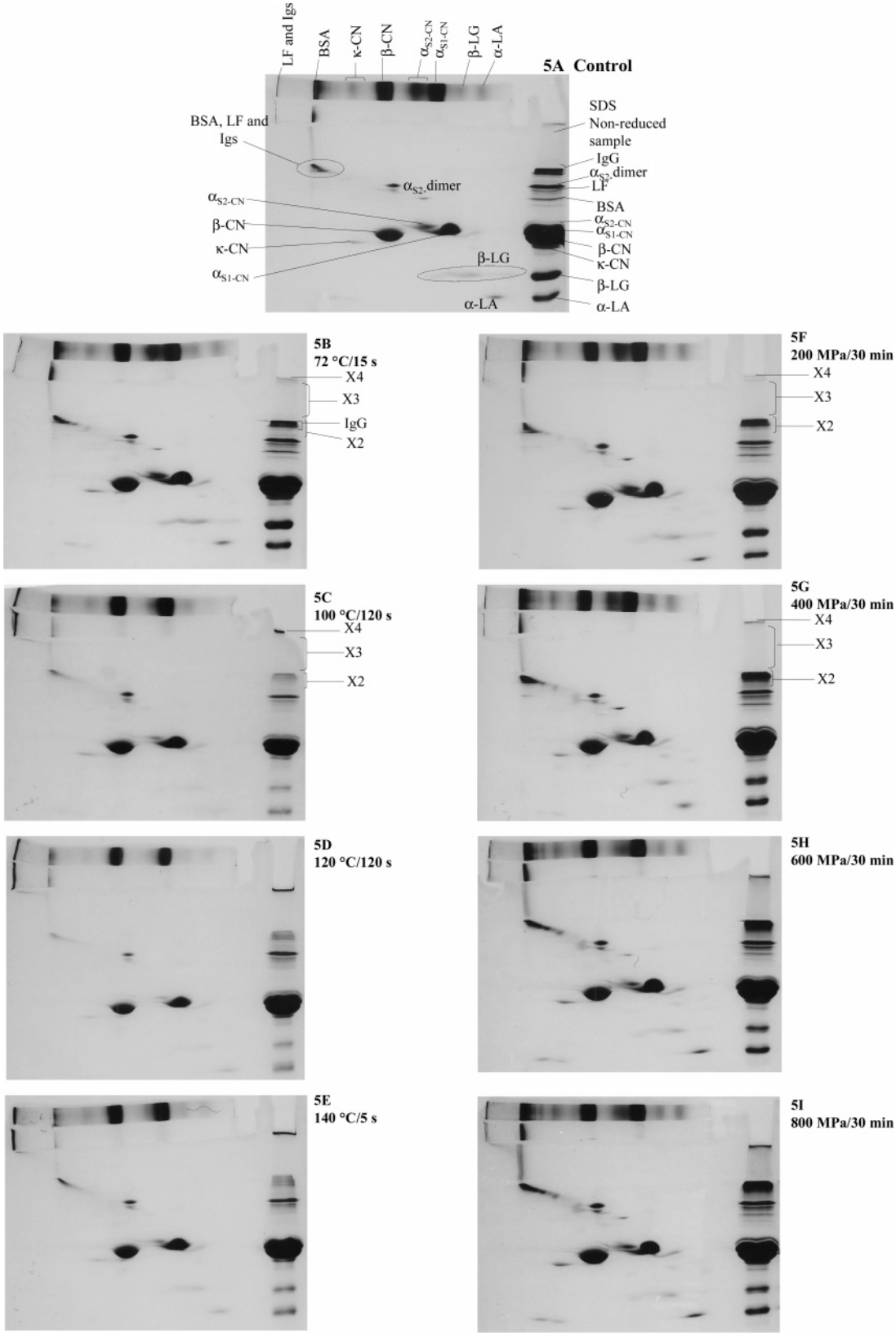

Figure 5. 2D AU- then nonreduced SDS-PAGE patterns of control (**A**) and heat-treated samples [72 °C for 15 s (**B**); 100 °C for 120 s (**C**); 120 °C for 120 s (**D**); 140 °C for 5 s (**E**)]; similarly, 2D PAGE patterns of pressure-treated samples [200 MPa for 30 min (**F**); 400 MPa for 30 min (**G**); 600 MPa for 30 min (**H**); 800 MPa for 30 min (**I**)].

proteins were the most affected. There were corresponding increases in the quantity of material in the X2, X3, and X4 regions. The change in α-LA concentration was slight, and the α_{s2}-CN dimer concentration seemed to be unaffected.

Urea-PAGE of Pressure-Treated Samples. Analysis of the same samples using urea-PAGE showed similar trends (**Figure 3E**). The pressure treatment diminished the quantities of monomer α_{s2}-CN and κ-CN in lanes 2−5, with corresponding

changes in the regions marked X2, X3, and X4. However, the differences were not as clear-cut as shown in the SDS-PAGE results (**Figure 3B**).

When the regions marked X2, X3, and X4 in the PAGE patterns of the heat- and pressure-treated samples were compared, it was found that the proportion of aggregates caught at the top of the resolving gel was comparatively higher in the PAGE patterns of the pressure-treated samples (**Figure 3B**,**E**, lanes 3−5), whereas the proportion of very large aggregates (that could not enter the gel or that were caught in the sample loading well) was comparatively higher in the PAGE patterns of the heat-treated samples (**Figure 3A**,**D**).

Comparison of the heat and pressure results showed that the severe heat treatments (e.g., **Figure 3A**,**D**, lanes 3−5) were more significant than the most severe pressure treatment (**Figure 3B**,**E**, lane 5).

2D PAGE. *SDS:SDS^R-PAGE of Heat-Treated Samples.* The untreated control sample of raw milk was analyzed by running the sample in a 1D SDS-PAGE gel; the strip was then cut and reduced with 2-ME and then set within the stacking gel of another SDS gel to run the reduced sample gel strip in SDS-PAGE in the second dimension (see **Figure 2** for a diagrammatic representation). The resultant SDS:SDS^R-PAGE gel pattern (**Figure 4A**) had a series of spots that lay diagonally from the lower right of the gel to the upper left. The proteins that gave rise to these spots had comparable mobility regardless of the action of 2-ME; that is, they had not been part of a disulfide-bonded protein aggregate. The order of decreasing mobility was γ-CN, α-LA, β-LG, κ-CN monomer, β-CN, α_{s1}-CN, α_{s2}-CN monomer, α_{s2}-CN dimer, and spots corresponding to some minor whey proteins (IgG, BSA, and LF). To the left of the diagonal, there was a small spot of reduced α_{s2}-CN and two significant streaks that were reduced κ-CN, indicating that they had been disulfide-bonded during the first dimension separation. The two κ-CN spots were resolved from the regions corresponding to X2 and X4 of the sample gel strip (a′) shown in **Figure 4A** (control) and **Figure 4B** (72 °C for 15 s). This indicates that much of the κ-CN was polymerized by disulfide bonds in the native state and that mild heat treatment (72 °C for 15 s) did not affect that distribution significantly. The three high-temperature treatments gave a significantly different pattern (**Figure 4C**−**E**), as compared with the control sample (**Figure 4A**), with all of the whey proteins, much of the κ-CN, and a small proportion of the α_{s2}-CN in a series of new bands that were resolved by reduction of protein aggregates caught in the set sample gel region (marked X4). The density of the α_{s2}-CN band (and the X4 sample gel) was greater in **Figure 4D** than in either **Figure 4C** or **4E**, showing that a heat treatment of 120 °C for 120 s was more effective than either of the other two high-heat treatments (100 °C for 120 s or 140 °C for 5 s) for incorporating α_{s2}-CN into the heat-induced disulfide-bonded aggregates. These results clearly explain that a combination of high temperature and longer holding time has severe effects on denaturation and aggregation of proteins as compared to short holding time.

SDS:SDS^R-PAGE of Pressure-Treated Samples. Comparison of the SDS- and then reduced SDS-PAGE patterns of pressure-treated (200 MPa for 30 min) skim milk (**Figure 4F**) with the untreated control (**Figure 4A**) showed that the pressure-treated sample contained less monomeric β-LG, a small quantity of dimer β-LG, and very small quantities of trimer, tetramer (labeled d_2, d_3, and d_4, respectively, on **Figure 4F**) and larger polymers of β-LG. Treatment at 400 MPa (**Figure 4G**) showed significantly greater loss of monomer β-LG and the presence of a significant number of bands that had been resolved from the X2 and X4 regions of the 1D SDS-PAGE pattern, indicating that the β-LG had been incorporated into moderately large disulfide-bonded aggregates that also included κ-CN and a minor band of monomer α_{s2}-CN as well. The sample treated at 600 MPa gave a similar pattern (**Figure 4H**) with more β-LG, κ-CN, and α_{s2}-CN as well as some α-LA from region X4 and less from region X2 or present as monomers or dimers. The same trend continued for the sample treated at 800 MPa (**Figure 4I**), with even more material in the set sample gel (X4) and less in the low-mobility region (X2) (**Figure 3B**) and with even more β-LG, α-LA, κ-CN, α_{s2}-CN, the minor whey proteins, and possibly α_{s2}-CN dimer in the large aggregates from regions X2 and X4.

Urea:SDS-PAGE of Control Sample. The untreated control sample of raw milk was analyzed by running the excised sample strip from the 1D urea-PAGE gel (**Figure 3D**, lane 1), which was then set into the stacking gel of an SDS gel (see **Figure 2**). The resultant urea:SDS-PAGE gel (**Figure 5A**) had two major spots near the center of the 2D PAGE pattern, which were identified as α_{s1}- and β-CN. Another almost oval spot adjoining the α_{s1}-CN spot was identified as monomeric α_{s2}-CNs (α_{s2}-CN 10P to α_{s2}-CN 13P) (*41*). The small intensely stained oval spot was dimeric α_{s2}-CN. κ-CN showed as a horizontal streak and contained both the A and B variants and their various post-translational modifications (*41*, *42*). β-LG showed as a horizontal streak, and α-LA, which also contained some glyco-α-LA, showed as a short horizontal streak under the β-LG streak (*31*). The minor whey proteins with high molecular weight were noted as BSA, LF, and Igs and formed a diagonal streak near the origin of the resolving gel region.

Urea:SDS-PAGE of Heat-Treated Samples. The 2D urea:SDS-PAGE pattern of pasteurized skim milk (72 °C for 15 s) (**Figure 5B**) was very similar to that of the control sample (**Figure 5A**). This result was not surprising as this was a relatively mild heat treatment. Both β-LG and κ-CN (monomer) were relatively unaffected, supporting the 1D PAGE results shown in **Figure 3A**.

The effects of all three severe heat treatments (100 °C for 120 s, 120 °C for 120 s, and 140 °C for 5 s) are shown (**Figure 5C**−**E**) and were similar to one another, although the intensities of both the monomer and dimer α_{s2}-CN spots were significantly diminished after a heat treatment of 120 °C for 120 s (**Figure 5D**), supporting the report that severely heat-treated milks (e.g., UHT-treated milk) contained β-LG:α_{s2}-CN complexes (*43*).

Urea:SDS-PAGE of Pressure-Treated Samples. The 2D urea:SDS-PAGE pattern of skim milk treated at 200 MPA for 30 min (**Figure 5F**) was similar to those of the control sample (**Figure 5A**) and the mildly heat-treated (72 °C for 15 s) skim milk sample (**Figure 5B**). The SDS-PAGE pattern at the right-hand side of each of the figures shows a dark band toward the top of the resolving gel, which was not observed with the patterns shown in **Figure 3D**,**E**, because the samples were not set in gels for the patterns shown in **Figure 3D**,**E**. We did not set the samples in the case of urea-PAGE, because we did not intend to reduce the aggregates in urea-PAGE. After pressure treatment at 400 MPa and higher (**Figure 5G**−**I**), the monomer α_{s2}-CN spot was less dense than in the control (**Figure 5A**). The intensity of this spot further diminished after pressure treatments of 600 MPa (**Figure 5H**) and 800 MPa (**Figure 5I**).

As the severity of pressure treatment increased, the density of the low-mobility diagonal streak increased, with corresponding decreases in the quantities of β-LG, κ-CN, and monomer α_{s2}-CN (**Figure 5F**−**I**). Because separation in a urea buffer

smears some of the protein bands, this particular technique is less useful for the whey proteins, but it is extremely valuable for determining the roles/fates of the monomer, dimer, and aggregates of α_{s2}-CN. It is clear that the quantity of monomer α_{s2}-CN gradually decreased with higher pressure treatments but that the quantity of dimer α_{s2}-CN did not change appreciably.

DISCUSSION

The composition of the casein:whey protein complexes formed during heat and pressure treatment of skim milk has not previously been studied in detail, partly because milk is a complex system in which all of its components, including the caseins, whey proteins, lactose, and milk salts, are initially in their natural form. In particular, the caseins are present as casein micelles in which the caseins are held together by a combination of hydrophobic association and cross-linking via small calcium phosphate clusters (*44*).

Method Development. In our previous studies on whey protein systems, 2D (native and nonreducing SDS) PAGE has proven to be a suitable method for analyzing heat-induced aggregates in model whey protein systems (*37*, *46*−*48*) and in WPC solutions (*30*, *31*, *38*).

However, some initial experiments showed that the 2D native:SDS-PAGE system using minigels (*31*) was less than optimal for the separation of the milk proteins because the strong casein:casein association in the native-PAGE system prevented sufficient differentiation of the caseins, in part because the relatively high ratio of casein to whey protein in milk made it difficult to observe the whey proteins in the presence of the casein bands that overloaded the PAGE system. In addition, the SDS-PAGE system (e.g., **Figure 3A**, lane 1), which worked well for whey protein samples (*31*), also poorly differentiated the SDS complexes of α_{s1}-CN, monomeric α_{s2}-CN, β-CN, and monomeric κ-CN because they had similar mobilities [which were not closely correlated to the molecular masses of the proteins (*49*)]. However, the 2D SDS:reduced SDS (SDS:SDS^R)-PAGE system (**Figure 4**) was suitable for showing that κ-CN was present in the large process-induced aggregates, which appeared as high molecular weight bands in the SDS dimension or as a series of spots in the SDS^R dimension.

Also in the SDS:SDS^R-PAGE pattern, for example, **Figure 4A**, monomeric α_{s2}-CN and κ-CN had mobilities similar to those of α_{s1}-CN and β-CN, respectively, and it was not easy to differentiate α_{s2}-CN monomer from α_{s1}-CN and κ-CN from β-CN, respectively. The introduction of urea into the sample to be analyzed and into the gel system (urea-PAGE) dispersed the caseins and separated them via their mobility at pH 8.5. Use of the SDS-PAGE method for the second dimension, for example, **Figure 5A**, separated the various proteins and their induced aggregates very effectively. This was particularly useful for analyzing the various α_{s2}-CN and κ-CN polymers and aggregates (**Figure 5H**) but less useful for analyzing native β-LG, because disulfide bond interchange was facilitated in the urea-PAGE environment (pH 8.6; 5 M urea) (**Figure 5A**), thus giving rise to streaks of polymerizing unfolded β-LG.

A second innovation was to set the sample in a large-pore polyacrylamide gel in the sample slot (**Figure 2**) [a procedure based on the concept of Davis (*40*) as discussed by Andrews (*50*)], to retain the aggregates during 2D electrophoretic analysis. When applied to the SDS:SDS^R-PAGE system (**Figure 4**), the material trapped in the 1D sample gel strip (including that trapped in the set sample gel region) was reduced and the individual proteins were resolved in the second dimension. The components of these large aggregates could then be determined,

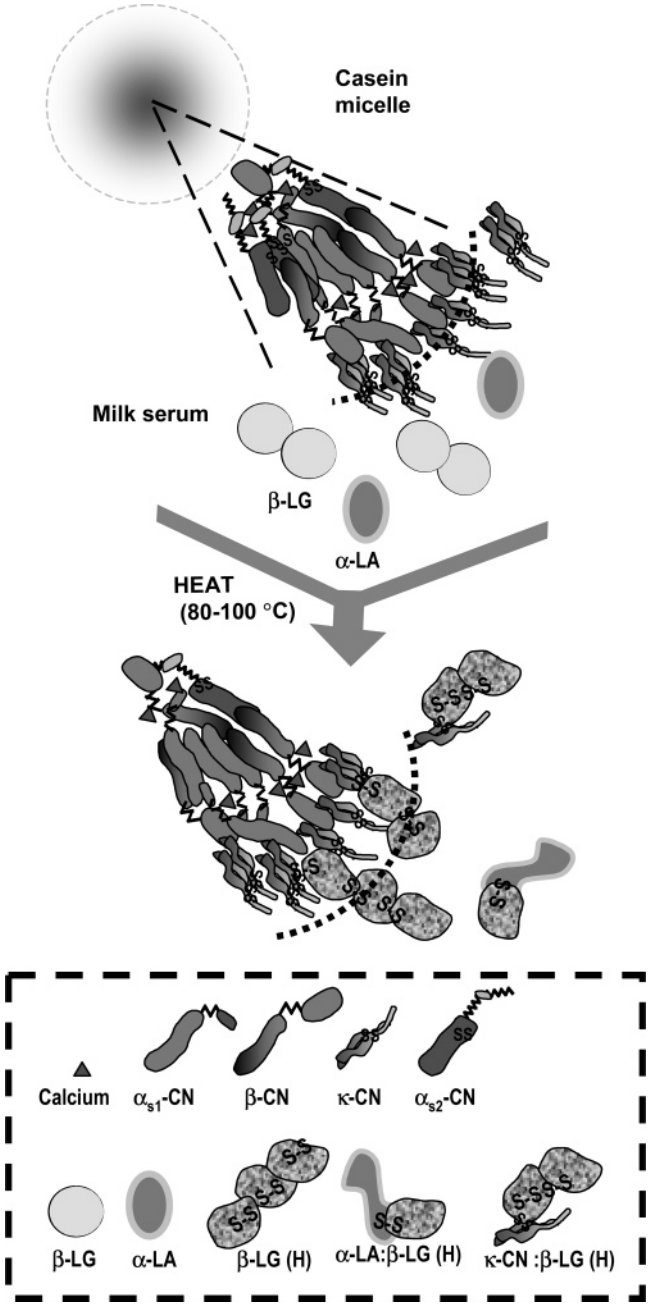

Figure 6. Pictorial representation of the likely effect of heating milk at 90 °C. The native β-LG dimer dissociates, and the monomer undergoes internal disulfide bond interchange to give reactive monomer polymers that react with κ-CN at the surface (outer region) of the casein micelle. Native β-LG monomers can also form an adduct with α-LA, which then gives rise to α-LA dimers and β-LG:α-LA dimers. In the severely heat-treated samples, α_{s2}-CN also forms disulfide bonds with other proteins.

thus showing the presence of both α_{s2}-CN and κ-CN, as well as β-LG and other whey proteins, in the pressure-induced aggregates (e.g., the series of new spots in the left-hand column resolved from region X4 on the sample gel strip of **Figure 4I**), and showed the relative incorporation of these proteins in the aggregates induced by heat and pressure treatments.

Process-Induced Aggregation. On the basis of our earlier studies of model systems (*9*, *37*, *47*, *48*, *51*−*54*) and WPC solutions (*30*, *31*, *38*, *55*), it is generally considered that, when milk is heated at ~85 °C, the major whey proteins (β-LG and α-LA) alter their structures and the free cysteine (CysH121) of β-LG initially reacts reversibly with the adjacent Cys106−

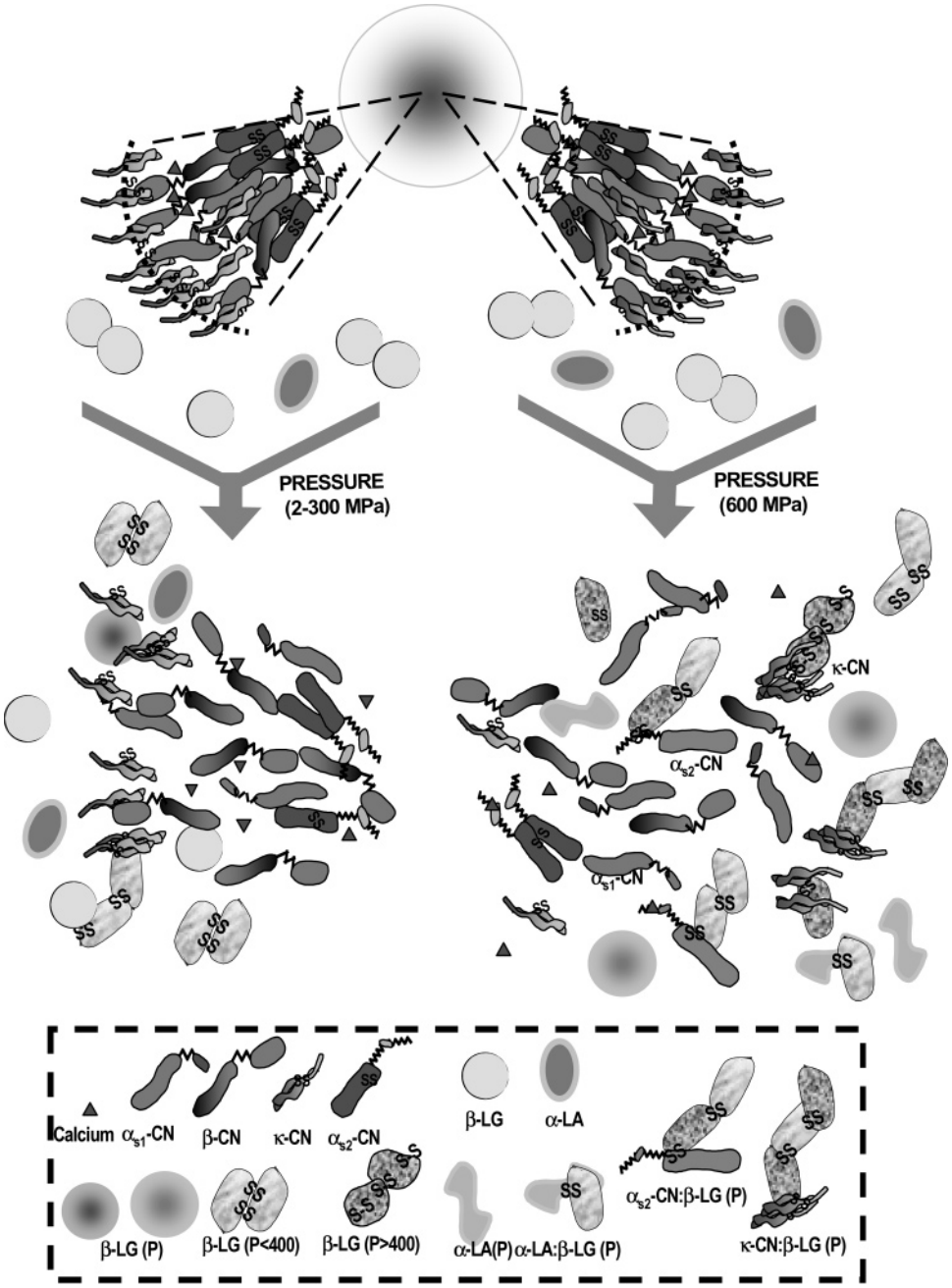

Figure 7. Pictorial representation of the likely effects of medium- (≈250 MPa) and high-pressure (>600 MPa) treatment at ≈22 °C. The casein micelle swells at ≈250 MPa, and the β-LG unfolds and aggregates via disulfide bonds. β-LG forms disulfide-bonded dimers at lower pressure and probably aggregates with κ-CN, but does not form larger β-LG aggregates. The proportion of α-LA that is included in the aggregates is less than that of β-LG (**Figure 3B**) because it does not readily unfold. At higher pressures, >600 MPa, α_{s2}-CN becomes available for thiol interchange reactions, assisted by the permeation of water into the micelle and the dissolution of the calcium phosphate. Also, the β-LG molecules can polymerize into aggregates larger than dimers,

Cys119 disulfide bond to give a free CysH119, which, in turn, reacts with the Cys66−Cys160 disulfide bond of the same or another (β-LG) molecule to give a free CysH160 (*52*). CysH160 is mobile and free to move because it is so close to the C terminus of the molecule. Thus, it can, and does, react with disulfide bonds in other proteins, allowing a chain reaction to occur with other β-LG (*52*) or κ-CN molecules (*53*).

When β-LG and α-LA are present together at high temperature, disulfide bond interchange can occur via a "molten globule" state (*56*−*58*), probably within a hydrophobic environment (*31*, *47*, *48*, *59*).

However, in the milk environment, the situation is different; that is, there are two caseins, α_{s2}-CN and κ-CN, that each contain two Cys residues: Cys36 and Cys41 for α_{s2}-CN and Cys11 and Cys88 for κ-CN (*41*). α_{s2}-CN is naturally present as a monomer (with one intramolecular disulfide bond) and as a dimer [with two intermolecular disulfide bonds (*60*−*62*)]. Although both forms are stable, the dimer appears to be comparatively more stable in heat- or pressure-treated milks (e.g., **Figure 4D**,**H**, respectively).

In contrast, κ-CN exists in milk as a wide range of κ-CN polymeric species ranging from monomers to large polymers (with intermolecular disulfide bonds), with many in the 6−12 monomers per aggregate range (*63*−*66*).

The virtual absence of α_{s2}-CN from the heat-induced aggregates formed at 85−90 °C in milk might be because α_{s2}-

CN is not a surface component of the micelle and therefore its disulfide bond(s) are inaccessible to the denaturing or denatured β-LG (**Figure 6**). On the other hand, κ-CN is on the surface of the micelles, and its disulfide bond(s) could be readily accessible to a thiol group from a denaturing β-LG. A diagrammatic representation of the consequences of the various interprotein reactions that take place during heat treatment of milk at ∼90 °C is shown in **Figure 6**. However, α_{s2}-CN is apparent in the large aggregates (regions X2 and X4) in milk samples heated at temperatures >100 °C (**Figure 4D**,**E**), supporting an earlier report by Snoeren and Van der Spek (*43*) and indicating that higher processing temperatures affect the proteins in a qualitatively different way.

In contrast, the effects of pressure treatment of milk (**Figure 7**) are different from those caused by heat treatment (**Figure 6**), mainly because pressure-induced changes to various milk proteins are quite different from the heat-induced changes of the milk proteins and their reactions.

Both the casein micelles (*69*) and the whey proteins (*36*) are compressed at pressures up to ∼150 MPa (*36*, *69*) and thereafter swell, as hydrogen bonds (which are important in maintaining helix and sheet structures) and hydrophobic associations are diminished and colloidal calcium phosphate dissolves. As a consequence, the micelles absorb more water, swell, and subsequently disperse (*36*, *69*).

The present results show that, at 200 MPa, β-LG does not appear to become involved in a chain reaction but is constrained to forming a stable, inactive, disulfide-bonded dimer [**Figure 7**, 2−300 MPa (*70*; Considine, Patel, Singh, and Creamer, unpublished data, 2005)]. At higher pressures (>400 MPa), the β-LG chain reaction becomes the norm and β-LG aggregation becomes similar to the heat-induced β-LG aggregation (**Figure 6**). This shows that the β-LG in WPC solutions (*30*, *31*) or in milk (present data) is not significantly modified by the other components; that is, β-LG dominates the denaturation and aggregation pathway during pressure (>400 MPa) treatment, as it has been shown to dominate high-temperature heat treatments (*31*, *67*, *68*) [**Figures 6** and **7** (>400 MPa)].

At low pressure, β-LG loses some peripheral turns and the native dimer dissociates into native monomers. At higher pressures, it loses the major helix, and then some of the sheet structures, and finally the F, G, and H motifs that are stable and contain the native Cys106−Cys119 disulfide bond, as discussed by Considine et al. (*67*, *68*) and Patel et al. (*31*). A free thiol also becomes available to interact with previously inaccessible disulfide bonds, and the intermediate species are probably very similar to the heat-induced early aggregates, but it is postulated that there is a stable disulfide-bonded dimer (as well as a reactive dimer) that is not readily transformed into larger disulfide-bonded aggregates (*67*, *68*, *70*; Considine, Patel, Singh, and Creamer, unpublished data, 2005) as indicated in **Figure 7** (<400 MPa).

The relatively lower degree of α-LA reactivity at high pressures is probably related to the relative stability of this protein compared with β-LG, as discussed by Patel et al. (*31*) and based on the unusual pressure-dependent behavior of α-LA (*58*, *71*, *72*).

Conclusions. Modifications to the previously used PAGE methods were invaluable in identifying the new disulfide-bonded species that were formed from the native proteins. Setting the samples in an open-pore gel (stacking gel mixture) prior to SDS-PAGE separation prevented the loss of the larger aggregates during the PAGE analysis procedure so that the protein components of these aggregates could be determined after disulfide bond reduction.

Consequently, it was possible to show that these large disulfide-bonded aggregates formed at high temperatures (>100 °C) or pressures (>400 MPa) contained complexes consisting of a high proportion of α_{s2}-CN as well as κ-CN, β-LG, and other whey proteins.

The addition of urea to native-PAGE gels and to the treated milk samples dissociated the casein molecules (and the processing-induced disulfide-bonded protein aggregates) from natural, or treated, micellar aggregates and the strong casein:casein hydrophobic associations, which allowed the α_{s2}-CN monomers and dimers to be identified and tracked.

The present study demonstrated that α_{s2}-CN as well as κ-CN aggregated with β-LG at high temperatures and at pressures >400 MPa. However, β-LG preferentially aggregated with κ-CN at 200 MPa. It was also concluded that dimer α_{s2}-CN was comparatively more stable than the monomer form after either high-pressure treatment or high-temperature treatment.

ABBREVIATIONS USED

1D, one-dimensional; 2D, two-dimensional; 2-ME, 2-mercaptoethanol; α-LA, α-lactalbumin; β-LG, β-lactoglobulin; κ-CN, κ-casein; AU, alkaline urea; BSA, bovine serum albumin; HP, high hydrostatic pressure; Ig, immunoglobulin; LF, bovine lactoferrin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SDS-PAGE, PAGE analysis in an SDS buffer; SDS^R-PAGE, SDS-PAGE of reduced samples; UHT, ultrahigh temperature; WPC, whey protein concentrate.

ACKNOWLEDGMENT

We thank Gerry Radford (Massey) for skilled assistance with the pilot plant trials and the high-temperature treatments and Claire Woodhall for editorial assistance with the manuscript.

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Received for review November 14, 2005. Revised manuscript received March 7, 2006. Accepted March 16, 2006. We are grateful to the New Zealand Foundation for Research, Science and Technology (Contracts DRIX0001 and DRIX0201) for funding this work.

JF052834C