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Pressure-Induced Unfolding and Aggregation of the Proteins in Whey Protein Concentrate Solutions

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Whey protein concentrate solutions (12% w/v, pH 6.65 ± 0.05) were pressure treated at 800 MPa for 20−120 min and then examined using size exclusion chromatography (SEC), small deformation rheology, transmission electron microscopy, and various types of one-dimensional (1D) and two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE). The pressure-treated samples showed a time-dependent loss of native whey proteins by SEC and 1D PAGE and a corresponding increase in non-native proteins and protein aggregates of different sizes. These aggregates altered the viscosity and opacity of the samples and were shown to be cross-linked by intermolecular disulfide bonds and by noncovalent interactions using 1D PAGE [alkaline (or native), sodium dodecyl sulfate (SDS), and SDS of reduced samples (SDS^R)] and 2D PAGE (native:SDS and SDS:SDS^R). The sensitivity of the major whey proteins to pressure was in the order β-lactoglobulin B (β-LG B) > β-LG A > bovine serum albumin (BSA) > α-lactalbumin (α-LA), and the large internal hydrophobic cavity of β-LG may have been partially responsible for its sensitivity to high-pressure treatments. It seemed likely that, at 800 MPa, the formation of a β-LG disulfide-bonded network preceded the formation of disulfide bonds between α-LA or BSA and β-LG to form multiprotein aggregates, possibly because the disulfide bonds of α-LA and BSA are less exposed than those of β-LG either during or after pressure treatment. It may be possible that intermolecular disulfide bond formation occurred at high pressure and that hydrophobic association became important after the high-pressure treatment.

KEYWORDS: Whey protein concentrate; β-lactoglobulin; protein aggregation; gelation; high hydrostatic pressure; 1D and 2D polyacrylamide gel electrophoresis; hydrophobic aggregation; disulfide-linked aggregates

INTRODUCTION

Interest in high hydrostatic pressure (HP) processing of dairy products has increased considerably in recent years (*1*−*3*), as it can be used to process food without thermal degradation and to create novel functionalities leading to new or improved products (*4*). HP can induce reversible and irreversible conformational changes to protein molecules, leading to protein denaturation, aggregation, or gelation (*5*) by way of polypeptide unfolding with consequential disulfide bond interchange (*6*−*8*).

Previous studies using raw milk (*9*−*11*), pasteurized milk (*12*), or reconstituted skim milk (*13*, *14*) concluded that α-lactalbumin (α-LA) was resistant to pressure denaturation up to 400 MPa for 60 min (*9*) or 500 MPa (*12*, *14*). Bovine serum albumin (BSA) and immunoglobulin (Ig) were resistant to denaturation up to 400 (*9*) and 300 MPa (*15*), respectively. β-Lactoglobulin (β-LG) seems to be the most sensitive of the major whey proteins and denatures at pressures as low as 150 MPa (*9*, *14*). These studies showed that the relative sensitivity of each whey protein in milk to HP treatment was different. Furthermore, it was confirmed that the major whey proteins in whey protein concentrate (WPC) solutions responded differently to heat and pressure treatments (*16*) and that the stability of β-LG and α-LA after pressure treatment was different in milk and in whey (*17*). It has been suggested, based on these results (*9*−*14*), that such differences in the denaturation and aggregation behavior could be due to differences in the protein structures and the stabilizing forces involved in maintaining the protein structures. These results also suggested that the whey proteins apparently act independently during the pressure treatment of milk and that the disulfides of α-LA are not accessible to the free thiol of β-LG, a very different situation from that in heated mixtures of α-LA and β-LG (*18*, *19*).

Studies have shown that different effects on proteins are observed when samples are analyzed under pressure and after pressure release. Many studies on the effects of high pressure

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on pure α-LA and β-LG solutions under pressure have been reported (*20*−*28*). The rate and the extent of modification were considerably increased by applying HP during the dansylation reaction of β-LG, whereas those for α-LA were only moderately affected. The fluorescence spectrum of these proteins measured under elevated pressure, as well as their fluorescence and circular dichroism (CD) spectra after pressure release, indicated different responses toward pressure (*29*). The structural change of α-LA was practically reversible up to 400 MPa, whereas that of β-LG lost reversibility at 150 MPa or lower. Fluorescent measurement of dansylated (prepared at atmospheric pressure) proteins, especially the energy transfer from the intrinsic Trp residue to the dansyl group, showed that the protein structure was deformed by pressure and that the energy transfer facilities of the two proteins were differently affected by high pressure, probably reflecting the degree of compactness of their pressure-perturbed structures (*20*, *29*). This indicated that β-LG unfolded in the 150−300 MPa range and that the unfolding was extensive and irreversible at pH 7 but much smaller and reversible at pH 3. It is likely that pressure unfolding at neutral pH enhances the reactivity of the SH group of β-LG and that intermolecular S−S bonds contribute to the irreversibility of unfolding. Belloque et al. (*25*) measured structural changes of β-LG during pressure treatments of 100, 200, or 400 MPa for 5 min in D_2O at neutral pH using ^1H nuclear magnetic resonance (NMR) on the acidified samples. They suggested that pressure-induced aggregates are formed by β-LG molecules maintaining most of their structure and that the intermolecular −SS− bonds, formed by −SH/−SS− exchange reactions, are likely to involve Cys66−Cys160 rather than Cys106−Cys119.

Previous work on the HP-induced denaturation and aggregation of β-LG and α-LA in a model system after pressure release showed that high-pressure treatment of β-LG solutions promotes unfolding and aggregation of β-LG through sulfhydryl−disulfide interchange reactions (*30*−*36*). Intermolecular interactions and irreversible aggregations are induced at high protein concentration (*37*, *38*) and, as a result of aggregation and gel formation, may lead to textural changes (*39*). β-LG is the major whey protein in WPC (*40*). Studies using WPC solutions have suggested that β-LG dominates the pressure-induced aggregation and gelation of WPC solutions (*25*, *41*−*43*), mainly through disulfide bonding with other whey proteins (*33*). More recently, Keim and Hinrichs (*44*) found that whey protein isolate (WPI) solutions (15% protein) formed stable gels after pressure treatment at 600 MPa, 30 °C, and holding times of up to 30 min. They showed that the content of native β-LG and α-LA decreased and that the proportion of intermolecular disulfide bonds increased with longer holding times and concluded that the number of stabilizing disulfide bonds directly influenced the textural properties of HP-induced whey protein gels. These studies (*30*−*44*) also indicated that the operating pressures, temperatures, protein concentrations, and treatment times appear to be important factors that influence the extent of whey protein denaturation, aggregation, and gelation.

It is evident from the available literature that most of the earlier work in the area of HP effects on milk proteins has been focused on the denaturation and aggregation of individual proteins, their denaturation kinetics, and rheological or microstructure analysis of whey protein gels. However, little is known about the pressure-induced interactions of the whey proteins in WPC solutions. Their detailed aggregation pathways and the interaction products that may be generated during pressure treatments have not been characterized in detail. Also, most of the earlier studies used pressures up to 600 MPa; the effects of 800 MPa for extended holding times on mixtures of whey proteins in WPC solutions have not been explored.

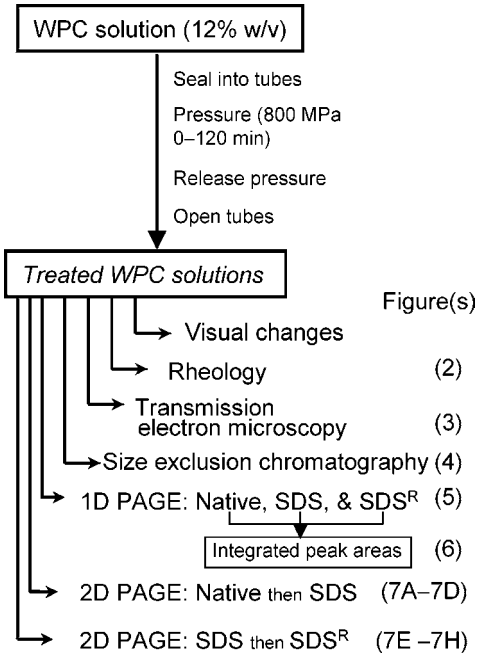

Figure 1. Summary of the experimental protocol used in this study.

In the present study, 12% WPC solutions were pressure treated at 800 MPa for up to 120 min and the resultant samples were characterized using rheological measurements, size exclusion chromatography (SEC), and transmission electron microscopy (TEM). The intermediate aggregates formed by the pressure treatment of WPC solutions were characterized using various one-dimensional (1D) and two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) techniques developed by Havea (*40*) to analyze the heat-induced aggregation of whey proteins in WPC solutions.

MATERIALS AND METHODS

The overall experimental plan is summarized in **Figure 1**.

Materials. A 25 kg bag of commercial acid WPC, obtained from NZMP, New Zealand, was used for the study. This WPC was typical of a standard commercial product. Analysis (*40*) showed that the WPC powder had 815 g/kg of total protein, 65 g/kg of fat, 45 g/kg of lactose, and 49 g/kg of moisture. Mineral analyses showed that the powder contained 1.7, 13.8, 8.0, and 2.5 g/kg of calcium, potassium, sodium, and phosphorus, respectively.

The major proteins of WPC were β-LG, α-LA, Igs, and BSA and were present in the ratio (w/w) 0.60:0.21:0.12:0.07 (*40*). These ratios change with milk source as well as milk and whey processing conditions. The proteins in WPCs were glycated with lactose as a consequence of processing (*45*, *46*), with α-LA and β-LG being the most sensitive (*47*, *48*). The type of WPC used in the current study typically contains β-LG that is attached to one lactose molecule (Higgs, K.; Norris, C.; Otter, D. Personal communication, 2000).

The WPC was subsampled and immediately stored at 4 °C in airtight, moisture-proof containers in order to prevent further changes. The electrophoresis chemicals and molecular weight markers 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 (BDH Laboratory Supplies, Poole, England). Water was purified by reverse osmosis treatment followed by carbon and deionization treatment using a Milli-Q apparatus (Millipore Corporation, Bedford, MA).

Preparation of WPC Samples. Aliquots of WPC solutions were prepared by dissolving appropriate quantities of acid WPC powder in Milli-Q water so that the final solutions contained 120 g of WPC per

liter of solution. The protein content of these solutions was about 98 g/L, assuming approximately 81.5% protein in the WPC. The solutions were stirred for 4−5 h at room temperature (22 ± 1 °C) using a magnetic stirrer to ensure complete dissolution. The pH of the solutions was measured after complete dissolution and was found to be in the range of 6.65 ± 0.05.

Pressure Treatment of WPC Samples. Beckman Polyallomer Quick-seal centrifuge tubes (13 mm internal diameter, 51 mm high, Beckman Instruments, Inc., Spinco Division, Palo Alto, CA) were filled with WPC solutions and heat sealed. The sample tubes were then treated in a high-pressure unit ("Food-Lab" food processor, model S-FL-085-9-W, Stansted Fluid Power Ltd., Stansted, Essex, United Kingdom) at 800 MPa and 22 °C for 20, 40, 60, 90, or 120 min. An emulsion of 10% vegetable oil homogenized in a solution of surfactant and preservative (Carroll, T., Fonterra Cooperative Group Ltd. Personal communication, 2003) was used as the pressure-transmitting fluid in the cylindrical 17 mm × 132 mm high-pressure chamber. The temperature of the pressure-transmitting fluid was maintained at 22 °C. The pressurization and depressurization rates were 5 and 14.5 MPa per second, respectively. The average adiabatic heating during pressurization was 1.4 °C/100 MPa. The cooling during depressurization was 1.3 °C/100 MPa. The temperature of the pressure-transmitting fluid returned to 22 °C in less than 5 min (*14*) after pressurization or depressurization. (The temperature of the pressure-transmitting fluid was measured in the center of the pressurizing chamber.) All of the samples in the present study were analyzed after pressure release, and no sample was analyzed under pressure.

Analysis of Pressure-Treated Samples. After each series of pressure treatments, the sample tubes were removed from the chamber and their contents were analyzed using a range of techniques.

Both the top and the bottom sections of the control and pressure-treated sample tubes were carefully cut using a sharp knife. The samples were carefully poured or slid from the tubes on to clean glass plates. The changes in the color and consistency of each sample were noted within 15 min of pressure release.

Each rheological analysis was completed within 6 h after pressure release. Sample fixing and preparation for TEM analysis were completed within 2 h of pressure treatment. The samples were diluted in native or sodium dodecyl sulfate (SDS) sample buffer for PAGE analysis within 1 h of pressure release, and then, the diluted subsamples were stored at −18 °C overnight and loaded on to the gels early the next day. The samples for SEC were prepared and stored at −18 °C and thawed just prior to SEC analysis.

Rheology. Rheological measurements, both rotational and dynamic, were made using a stress-controlled Paar Physica rheometer model UDS200 (Paar Physica, Stuttgart, Germany), with a cone−plate geometry at a constant temperature of 20 °C. The cone had a diameter of 50 mm and an angle of 2°. The flow curves were obtained in a steady state mode, and the frequency sweep measurements were obtained at a constant strain of 10%.

TEM. The control and pressure-treated WPC samples were fixed (within 2−3 h after depressurization), stained, and processed as described by Langton and Hermansson (*49*), and the TEM images were recorded photographically.

SEC. The control and pressure-treated WPC samples were diluted by mixing 1 part of sample with 19 parts (w/w) of the elution buffer (20 mM imidazole/HCl, 50 mM NaCl, pH 6.80). The samples that had been pressure treated for more than 60 min were difficult to disperse and often required extensive stirring and agitation using a vortex mixer. The solutions were then filtered through a 0.22 μm membrane filter (Millipore Corporation). These samples were then analyzed using a high-performance liquid chromatography (HPLC) system, Aligent model 1050 (Aligent Technologies, Palo Alto, CA), fitted with an Aligent 1050 pump and an Aligent 1050 detector set. The HPLC column [150 mm × 30 mm TOSOH TSK-G4000$_{PWXL}$ (Supelco, Bellefonte, PA)] was equilibrated with the elution buffer at a flow rate of 0.2 mL/min. The samples were loaded and eluted at 0.2 mL/min, and the eluates were monitored at 214, 280, and 320 nm.

Preparation of Subsamples for PAGE Analysis. Small quantities (50 ± 1.0 mg) of each of the control and pressure-treated samples were weighed accurately into Eppendorf tubes and dispersed into 1.0 mL of native or SDS gel sample buffer and mixed using a vortex mixer. The samples that had been pressure treated for 90 min or longer were stirred intermittently with a vortex mixer and often took 16 h to dissolve. A set of SDS-reduced samples was prepared by heating a 1 mL aliquot of SDS subsample (control and pressure-treated samples) with 20 μL of 2-ME at 94 °C for 4 min.

1D and 2D PAGE. The control and pressure-treated samples were analyzed using a Mini-Protean II dual cell system (Bio-Rad Laboratories) and the discontinuous PAGE system for both 1D and 2D PAGE. The methods described by Havea et al. (*50*) were used for 1D and 2D PAGE analysis. The gels were scanned and photographed, as described by Manderson et al. (*51*), using a computing laser densitometer (Molecular Dynamics model P. D., Sunnyvale, CA), and the integrated intensities of the β-LG, α-LA, and BSA bands were determined using Molecular Dynamics ImageQuant software (Version 5.0). The experiment was repeated using similar experimental conditions, and the variations in the integration results were found to be within 5−10%. The data presented in **Figure 6** are the averages of two integrations.

RESULTS

Visual Changes. When the WPC solutions were pressure treated at 800 MPa, the color changed from opalescent pale dun to pale yellow and finally to whitish-yellow. The clarity of the samples decreased initially and then increased with longer pressure treatments. After a pressure treatment of 800 MPa for 20 min, the sample consistency was almost unchanged. The consistency of the samples increased with the severity of pressure treatment, and the samples became viscous after a pressure treatment of 60 min. After 90 min of pressure treatment, the samples were quite viscous. The solutions were transformed into stable, elastic, almost translucent weak gels after 120 min of pressure treatment.

Rheology. The viscosity as a function of shear rate for the control and the sample pressure treated for 20 min remained independent of the shear rate (Newtonian behavior) (**Figure 2A**). For longer pressurization times, the pressurized samples deviated from Newtonian behavior, to exhibit pseudo-plastic behavior. Furthermore, the viscosity and the extent of pseudo-plasticity of these samples increased with an increase in pressurization time (see **Figure 2A**, inset). To quantify the extent of pseudo-plasticity, the viscosity η (Pa s) was calculated as a function of the shear rate $\dot{\gamma}$ using a power law equation of the form $\eta = k\dot{\gamma}^{n-1}$ where k is the consistency index and n is the flow behavior index, which is related to the extent of pseudo-plasticity (for a Newtonian liquid, $n = 1$). It was found that η was equal to 0.32, 0.70, and 0.96 for 20, 40, and 60 min of pressurization time, respectively.

In addition to the increase in pseudo-plasticity and viscosity, the pressure-treated samples also became elastic. The frequency sweep measurement showed that, for pressure treatment times longer than 40 min, the elastic modulus G' was higher than the viscous modulus G'' (**Figure 2B**). In addition, the elasticity of these gels increased exponentially with the time of pressure treatment (**Figure 2B**, inset). However, even after a longer pressure treatment time (90 min), these samples still behaved as weak gels. To further illustrate the effect of high pressure on the viscoelasticity of these samples, tan δ (G''/G') is reported in **Figure 2C**. It was observed that the sample pressurized for 40 min remained mainly liquid up to a frequency of 1 Hz, with a tan δ value close to 1, and started to become elastic at higher frequencies. However, for samples pressurized for 60 and 90 min, tan δ was less than 0.45, which indicated the elastic nature of these samples.

TEM. Two sets of pressure-treated samples were fixed, stained, and examined using TEM, and a typical set of micrographs is shown in **Figure 3A−F**. The untreated control

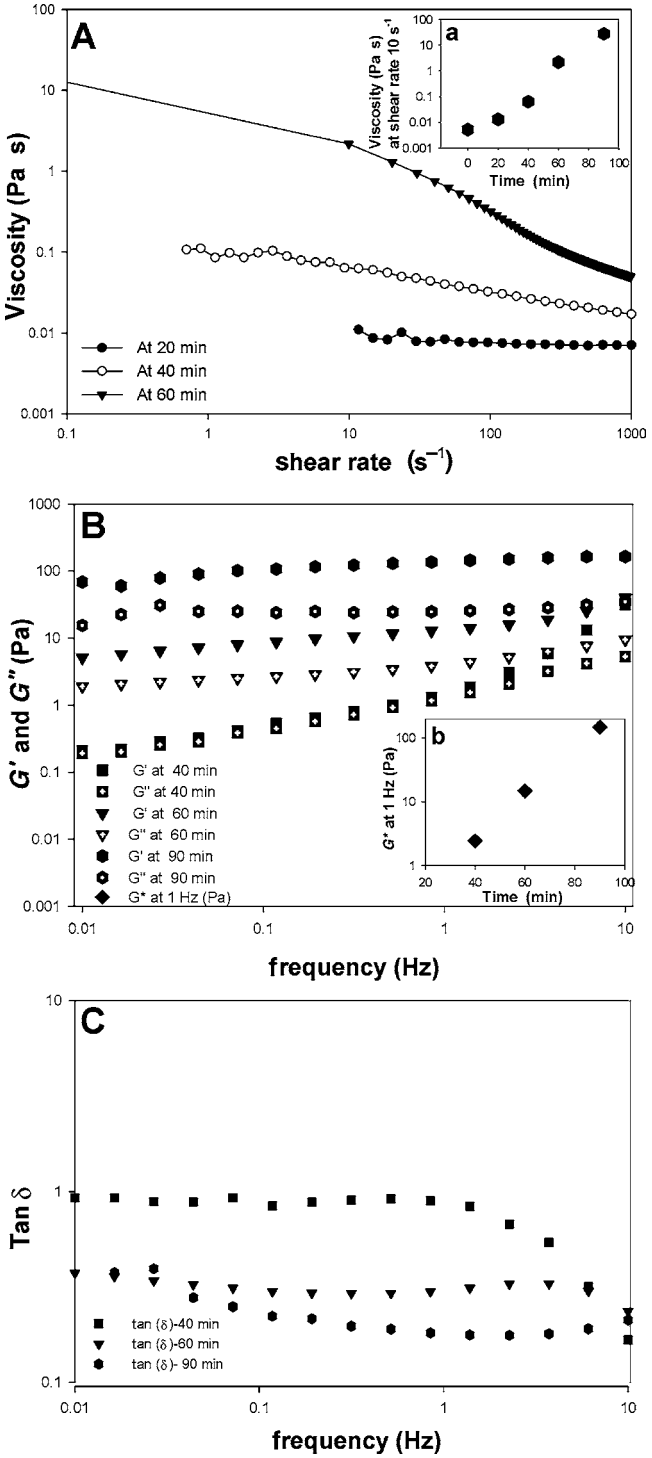

Figure 2. Effect of pressure treatment (800 MPa at 22 °C) for (**A**) 20, 40, and 60 min on the shear rate dependence of the sample viscosity measured at 20 °C and for (**B**) 40, 60, and 90 min on the frequency dependence of G' and G''. Inset **a** shows the viscosity for the samples pressure treated for 20, 40, 60, and 90 min at a shear rate of 10 s^{-1}. Inset **b** shows G^* for the samples pressure treated for 40, 60, and 90 min at 1 Hz. (**C**) Calculated tan δ (G''/G') of pressure-treated samples at different frequencies.

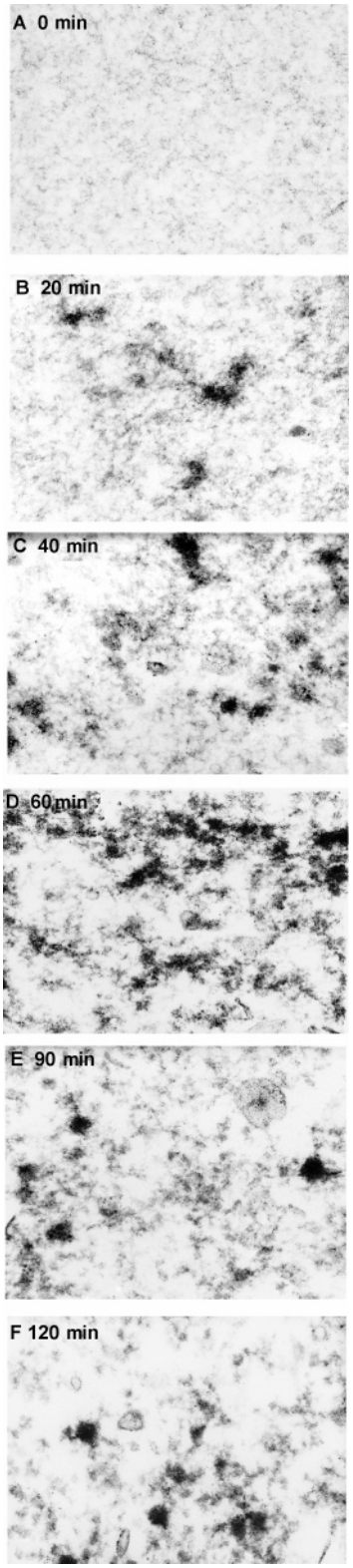

Figure 3. TEMs of WPC solutions (12% w/v) pressure treated (800 MPa at 22 °C) for (**A**) 0, (**B**) 20, (**C**) 40, (**D**) 60, (**E**) 90, and (**F**) 120 min. After pressure treatment, the individual sample was removed, fixed, stained, and photographed.

sample (**Figure 3A**) did not show any sign of aggregated proteins. The WPC sample that was pressure treated (800 MPa) for 20 min (**Figure 3B**) showed the formation of fine hairlike strands of almost uniform size. These fine strands were distributed randomly throughout the sample. The structure of these fine strands appeared to be comparable with the "fine-stranded" structures of heat-induced β-LG gels (12% w/v, pH 7.50) reported by Stading and Hermansson (*52*). In addition, some clumps or aggregates of strands were observed after a pressure treatment of 20 min.

The aggregates were larger after 40 min of pressure treatment (**Figure 3C**). There were more aggregates of about the same

size after 60 min of pressure treatment, and some appeared to be linked to give even larger aggregates (**Figure 3D**). Longer pressure treatment times (**Figure 3E**,**F**) did not appear to increase the number or size of the aggregates, but there appeared to be more low-density areas. This "particulate" material appeared to be similar to that observed in the heated whey protein system reported earlier (*49*, *52*) and appeared to be consistent with earlier scanning electron micrographs of pressure-treated WPC solutions (*34*, *35*). These results also compared well with those of Walkenström and Hermansson (*53*), who reported that, at pH 6.8, the mixed gel formed a phase-separated system, composed of a network of aggregates and a phase with fine strands, studied in a mixed gelatin and WPC system. Overall, it appeared that particulate aggregate structures were formed as a consequence of 40−60 min of pressure treatment at 800 MPa, but there appeared to be no further increase in particle size or numbers after longer pressure treatments (**Figure 3D**−**F**) despite the changes in the rheological data (**Figure 2**).

SEC. The untreated sample gave a major peak eluting at about 29 min with a minor peak eluting at 31 min (**Figure 4A**). The major components of WPC (β-LG and α-LA) were the likely source of the major peak. The minor peak at 31 min (**Figure 4**) was probably orotic acid because it showed a substantial absorbance at 320 nm (Elgar, D., Fonterra Cooperative Group Ltd. Personal communication, 1999). After pressure treatment, the samples showed a marked and continuing decrease in the size of the major peak and increased absorbance between 19 and 27 min of elution time. For the 20 min sample (**Figure 4B**), there was a small broad peak at about 26 min, which corresponded to β-LG dimers (*54*). There appeared to be a peak with an elution time of about 21 min in the 40 min sample (**Figure 4C**), but this void volume "peak" contained material that was too large to enter the gel pores and small enough to pass the filter (0.22 μm) and enter the column. In the region between 22 and 25 min, the absorbance corresponded to intermediate-sized aggregates, which would probably be seen as low-mobility staining on PAGE patterns (e.g., **Figure 5A**, lanes 2−5). As the pressure treatment time increased, the total quantity of material that entered the column decreased and the height of the 29 min peak after 120 min (**Figure 4F**) was about 10% that of the control (**Figure 4A**), suggesting that at least 90% of the original native protein had been denatured and polymerized to give very large aggregates that would not pass a 0.22 μm filter to enter the column. The resolution of the protein peaks in the present study was less than that obtained using a Superdex-75 column in an earlier study (*54*).

1D PAGE Analysis. The PAGE patterns of control and pressure-treated WPC samples, analyzed by 1D native-, SDS-, and SDS of reduced samples (SDS^R) PAGE, are presented in **Figure 5A**−**C**, respectively. The identities of most protein bands in the control sample were determined by comparison with the results of Havea et al. (*50*), and the positions of the major whey proteins are noted on the left-hand side of the PAGE patterns. Pressure-induced aggregates are denoted as X_1−X_6 and $\underline{X}_2$−$\underline{X}_6$ on the right-hand side of the native- and SDS-PAGE patterns, respectively (**Figure 5A**,**B**, respectively).

Native PAGE. The native PAGE patterns of the pressure-treated WPC solutions (**Figure 5A**) showed that the quantity of nativelike monomeric whey proteins decreased with increasing pressure treatment time. It was clear from the PAGE pattern that the WPC used in the present study contained small amounts of glyco-α-LA (denoted as α-LA*), the details of which are included in the Materials and Methods section. However,

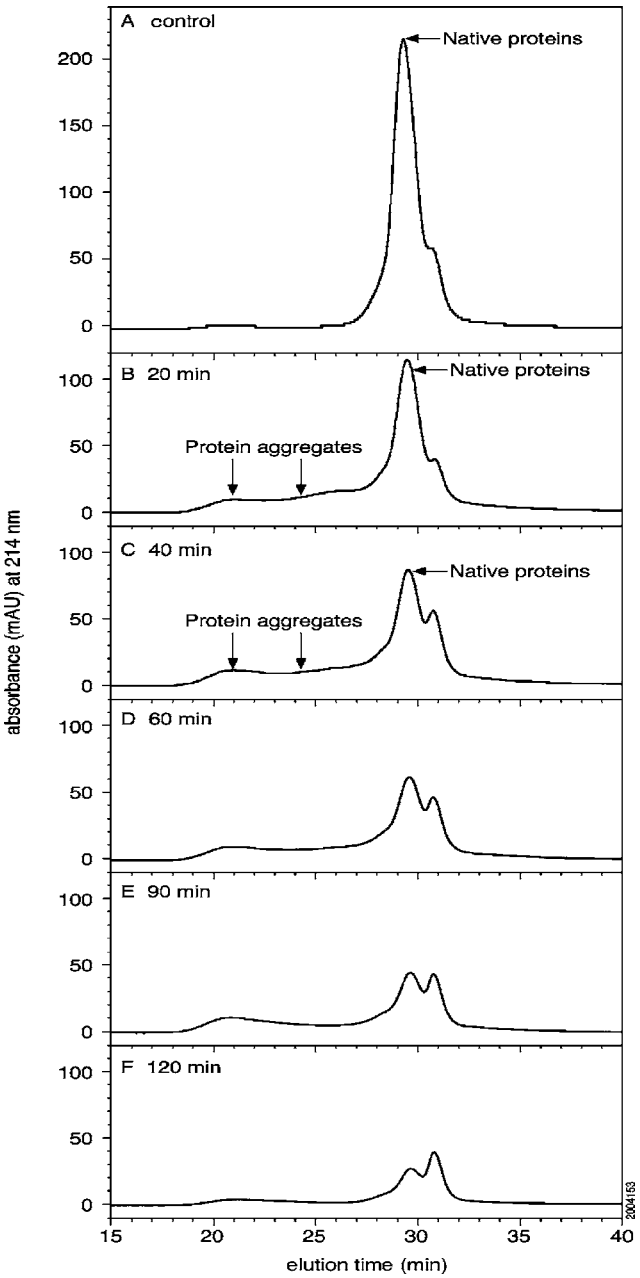

Figure 4. SEC patterns of WPC solutions (12% w/v) pressure treated (800 MPa at 22 °C) for (**A**) 0, (**B**) 20, (**C**) 40, (**D**) 60, (**E**) 90, and (**F**) 120 min and monitored at 214 nm.

dimeric β-LG (β-LG_2), caseins, and lactosylated adducts of β-LG were not identified clearly in the control sample. The non-native β-LG species that are apparent in pure β-LG systems (*55*, *56*) were also not seen clearly in the PAGE patterns, probably because they were obscured by the other protein species.

The A and B variants of β-LG were partially separated from one another, and the quantity of β-LG B decreased faster than that of β-LG A (**Figure 5A**, lanes 3−6). This was much clearer when lower loadings were used for the PAGE analysis (results not shown). The intensities of the nativelike β-LG A, β-LG B, α-LA, and BSA bands decreased with more extensive pressure treatment. The quantified data were plotted against pressure treatment time (**Figure 6B**). The rate of loss of the three major whey proteins was β-LG > BSA > α-LA. This trend was quite different from that obtained for the heat treatment of WPC

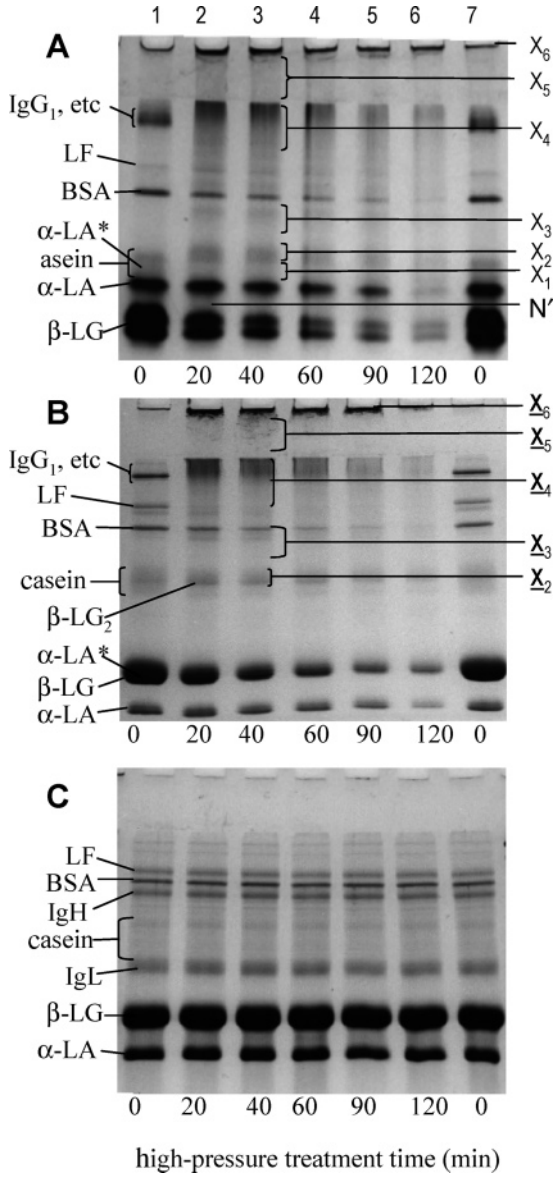

Figure 5. Typical 1D (**A**) native-, (**B**) SDS-, and (**C**) SDS^R-PAGE patterns of pressure-treated (800 MPa for 0–120 min at 22 °C) WPC solutions. Lanes 1 and 7, control samples. For the pressure-treated samples: lane 2, 20 min; lane 3, 40 min; lane 4, 60 min; lane 5, 90 min; and lane 6, 120 min. The identities of the major protein bands in the control samples are noted on the left-hand side, and the new protein bands present as a consequence of the pressure treatment are denoted as X_1–X_6 on alkaline-PAGE (**A**) and as $\underline{X}_2$–$\underline{X}_6$ on SDS-PAGE (**B**). See the text for the experimental details and detailed identifications of the various bands in the control sample and the pressure-treated samples. The abbreviations for the proteins and the experimental conditions are also described in the text.

solutions (BSA > β-LG ≈ α-LA), using comparable analytical methods (*50*).

Bands X_2 and X_3 (**Figure 5A**, lanes 2−6), which were not present in the control sample, mostly corresponded to β-LG dimers and trimers, respectively. These species were also identified using 2D PAGE techniques (compare with **Figure 7**). These bands (X_2 and X_3) appeared to reach maximum concentrations in the sample that was pressure treated for 40 min (**Figure 5A**, lane 3), and thereafter, their intensities decreased. Similarly, bands X_4 and X_5 reached maximum intensities in the sample that was pressure treated for 60 min

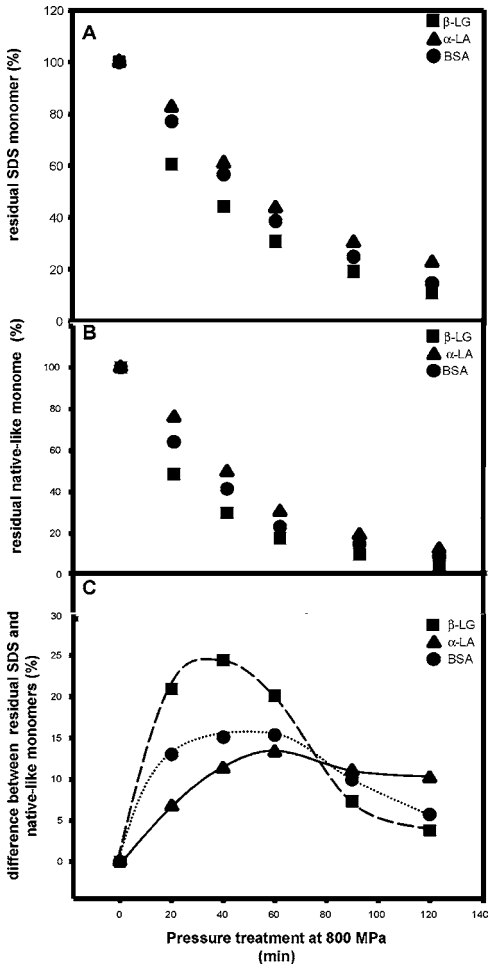

Figure 6. Percentage of (**A**) residual SDS-monomeric and (**B**) residual nativelike monomeric β-LG, α-LA, and BSA as a function of the pressure treatment time. (**C**) The differences in the residual SDS-monomeric and nativelike monomeric bands as a function of the pressure treatment time.

(**Figure 5A**, lane 4). The lower intensity of band X_6 in the samples pressure treated for 90 min and longer (**Figure 5A**, lanes 5 and 6) indicates that the aggregates were too large to enter the stacking gel or to remain attached in the sample loading well of the stacking gel and therefore were often lost during the staining and destaining procedures. These results suggest that the pressure-induced reactions progressed toward the formation of larger and larger aggregates.

The largest aggregates on the top part of the stacking gel or caught in the sample well (X_6), within the stacking gel (X_5), and at or near the top of the resolving gel (X_4) probably consisted of both disulfide-linked and noncovalently associated proteins and contained protein aggregates of low net negative charge and/or high molecular weight.

SDS-PAGE. The major protein bands were also readily identified in the SDS-PAGE pattern of the control sample (**Figure 5B**, lane 1). The A and B variants of β-LG were indistinguishable and migrated more slowly than α-LA, in contrast to their mobility in the native PAGE pattern (**Figure 5A**).

The regions labeled $\underline{X}_2$−$\underline{X}_6$ (**Figure 5B**) indicate the positions of new bands that were formed as a consequence of the pressure treatment. The largest aggregates were at the top of the stacking gel or in the sample loading well ($\underline{X}_6$), within the stacking gel ($\underline{X}_5$), and at or near the top of the resolving gel ($\underline{X}_4$). These aggregates did not contain noncovalently associated proteins because of the dissociating power of the SDS in SDS-PAGE.

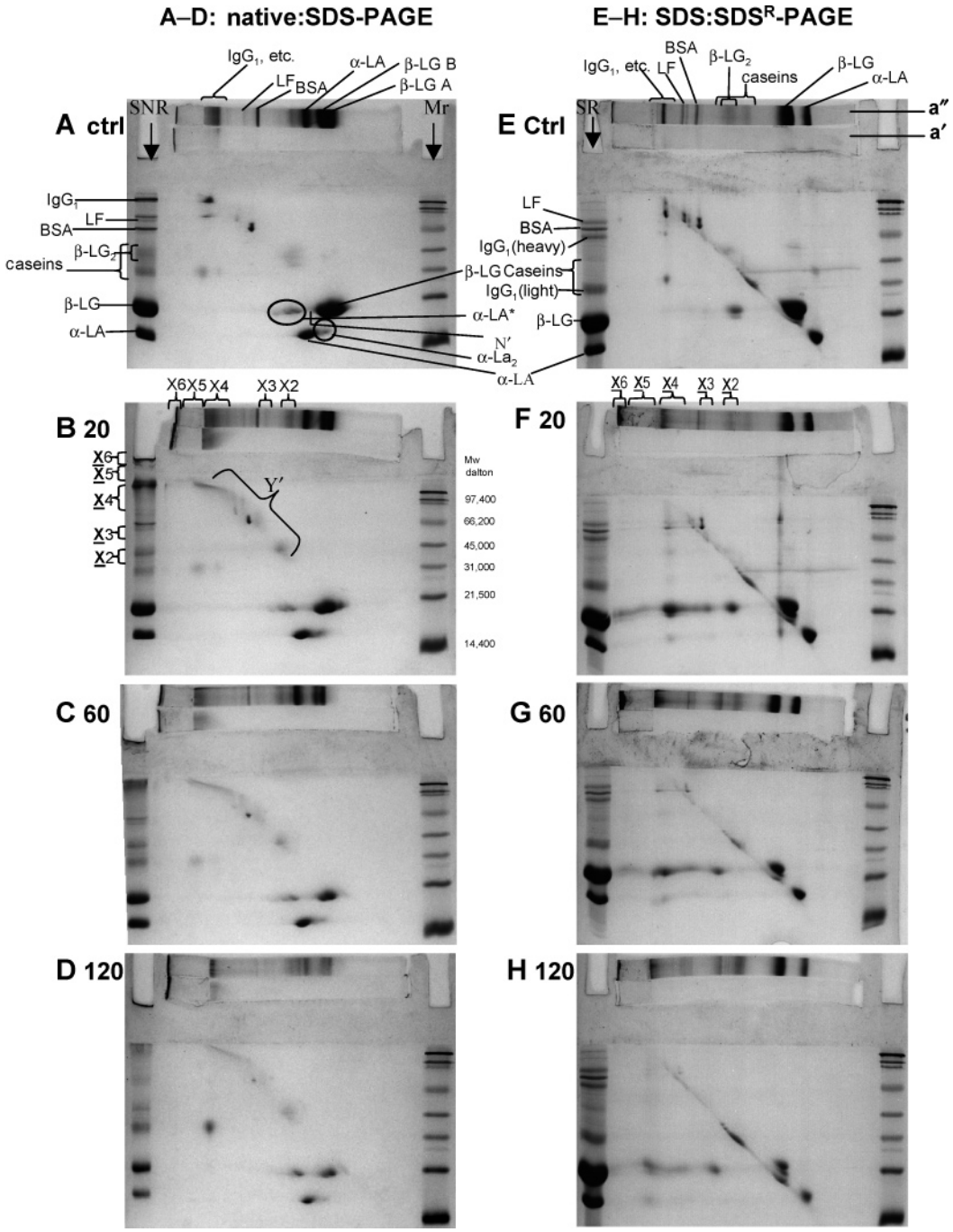

Figure 7. 2D PAGE patterns of control and pressure-treated WPC solutions (12% w/v). Native- and then nonreduced SDS-PAGE patterns of (**A**) the control and samples pressure treated for (**B**) 20, (**C**) 60, and (**D**) 120 min. Similarly, SDS- and then SDS^R-PAGE patterns of (**E**) the control and samples pressure treated for (**F**) 20, (**G**) 60, and (**H**) 120 min. See the text for the experimental details and identifications of the various protein spots in the control sample and of the interaction products in the pressure-treated samples.

Bands $\underline{X}_2$ and $\underline{X}_3$, which were not present in the control sample, corresponded to newly formed β-LG dimers and trimers and were clearly identified as disulfide-linked dimers and trimers using 2D SDS:SDS^R-PAGE (**Figure 7E**−**H**). These bands appeared to attain maximum intensity at between 20 and 60 min of pressure treatment (**Figure 5B**, lane 4).

SDS^R-PAGE. Reduction of the control and the pressure-treated samples prior to analysis by SDS-PAGE gave almost identical patterns (**Figure 5C**), showing that there were no pressure-induced chemical changes leading to the formation of aggregates that could not be reduced using 2-ME.

Formation of Covalent and Noncovalently Linked Protein Aggregates. The percentage residual nativelike and SDS monomeric β-LG, α-LA, and BSA after pressure treatment are shown in **Figure 6B**,**A**, respectively. The normalized intensities of the various bands are presented as a proportion of the band intensities of the control sample. Clearly, the proportion of monomer β-LG decreased faster than that of α-LA or BSA (**Figure 6A**,**B**), supporting the findings of earlier comparable studies (*16*) that β-LG was more sensitive to HP treatment than α-LA or BSA. The changes in the quantities of β-LG in the native and SDS-PAGE patterns were not the same because of the presence of non-native β-LG, which may be an intermediate in the aggregation process; non-native β-LG migrates in SDS-PAGE as a monomer but migrates more slowly than the native monomer in native PAGE. Quantitative analysis of the protein

bands of the control and pressure-treated samples in the 1D native and SDS-PAGE patterns (**Figure 6B**,**A**) showed that the loss of nativelike proteins (**Figure 6B**) was comparatively greater than the loss of SDS monomeric proteins (**Figure 6A**) for all whey proteins, including β-LG, α-LA, and BSA. The difference in the quantities of the monomers, assessed by PAGE analysis, has been called the "non-native monomer" species and has been investigated and discussed (*50*, *51*, *55*−*57*). The values plotted in **Figure 6C** are the differences between the quantities of residual SDS monomeric proteins (**Figure 6A**) and the quantities of residual nativelike proteins (**Figure 6B**) present in the WPC solutions after various pressure treatments. These results give some indication about changes in non-native β-LG, α-LA, and BSA with pressure treatment. Most of the β-LG was converted into disulfide-bonded dimers, trimers, etc. (see **Figure 7F**), but some was converted into non-native monomeric β-LG (**Figure 6C**). These results indicated that approximately 5−25% of the aggregates formed during the pressure treatment of WPC solutions were noncovalently linked (**Figure 6C**) and that the majority (approximately 75−95%) of these aggregates were linked by disulfide bonds.

Characterization of Pressure-Induced Protein Interactions Using 2D PAGE. The WPC samples were further analyzed using 2D PAGE techniques (native:SDS- and SDS:SDS^R-PAGE), to identify the intermediate aggregates formed during pressure treatment. The general procedures that have been used previously by our group to characterize heat-induced (*50*, *58*, *59*) or pressure-induced (*16*) aggregates in WPC solutions were followed to give the results shown in **Figure 7**.

In the 2D native:SDS-PAGE procedure, a sample is analyzed using 1D PAGE in a Tris HCl buffer at pH 8.7 and called alkaline-PAGE, and the gel strip with its separated protein bands is transferred to SDS-PAGE in the second dimension (i.e., transferred into a dissociating environment). Once the proteins in the strip are partially equilibrated with the SDS to form SDS−protein complexes, they are electrophoresed into a new (SDS) environment in a second dimension PAGE analysis. This combination of native and then nonreduced SDS-PAGE disrupts noncovalent bonding, whereas covalent bonds remain unaffected.

In the 2D SDS:SDS^R-PAGE procedure, the protein components in the sample are separated on the SDS-PAGE gel, the proteins are reduced with excess 2-ME while they are still in the gel strip, and the components are identified after an SDS-PAGE analysis in the second dimension. This combination disrupts the disulfide-linked protein aggregates.

2D Native:SDS-PAGE. The PAGE patterns of the control and the samples that were pressure treated for 20, 60, or 120 min are shown in **Figure 7A**−**D**, respectively. Each figure contains the horizontally mounted stained gel strip (marked as **a**″), the sample gel strip (marked as **a**′) from the 1D alkaline-PAGE, the corresponding SDS-PAGE pattern of the nonreduced sample in the left-hand lane (denoted as SNR), and a series of molecular weight standards in the right-hand lane (denoted as Mr).

Control Sample. The β-LG, α-LA, α-LA*, BSA, and lactoferrin (LF) monomers identified earlier in WPC samples (*50*) can be readily seen in **Figure 7A**. The spot adjoining to α-LA could be an α-LA dimer (marked as α-LA$_2$) (*19*, *60*), which may be present in WPC as a consequence of processing. The bands close to the top of the resolving gel (in the 1D alkaline-PAGE sample gel strip) were resolved into three spots in the second dimension. One spot was identified as IgG$_1$ (*61*, *62*), another was probably LF, and the third, which had the mobility of a protein with an apparent molecular mass of about 35000 Da, was probably an aggregated casein, most likely κ-casein that had been hydrophobically aggregated in the absence of SDS (in the first dimension alkaline-PAGE). There were a number of diffuse spots near the center of the 2D gel, probably corresponding to a range of dimeric β-LG and various casein fractions.

Pressure-Treated Samples. Comparison of the 2D PAGE pattern of the control sample (**Figure 7A**) with those of the pressure-treated samples (**Figure 7B**−**D**) showed that the intensity of all monomeric whey protein spots, including β-LG and α-LA, decreased with the time of pressure treatment, with the simultaneous appearance of new protein spots (as shown in the region Y′, **Figure 7B**−**D**). The protein spots corresponding to monomeric Igs and LF were absent from the samples that were pressure treated for >40 min (**Figure 7C**,**D**), but a faint spot of monomeric BSA was still observable even after 120 min. Comparison of the sample gel strip (**a**′) in the 2D gel with the stained gel strips (**a**″) of the control sample (**Figure 7A**) and the pressure-treated samples (**Figure 7B**−**D**) showed that the high molecular weight protein aggregates formed by pressure treatment (**Figure 7B**−**D**, particularly X$_4$−X$_6$ on the stained gel strip) were only partially dissociated by SDS in the second dimension. Some protein aggregates (marked X$_2$, X$_3$, X$_4$, **Figure 7B**) dissociated from the region between the α-LA band and the start of the resolving gel of the sample gel strip to give many closely located protein spots on the 2D gels, forming an almost continuous faint inclined line (marked Y′, **Figure 7B**). These results confirmed that some of these aggregates were noncovalently linked. The band with a mobility intermediate between monomer and dimer in the first dimension (**Figure 5**) had the same electrophoretic mobility in the second dimension as the monomer (**Figure 7A**, N′) and therefore was identified as non-native monomer by Manderson et al. (*51*). Also, there was a series of closely located spots in the region between the β-LG spot and the β-LG band in the left-hand lane. These spots corresponded to hydrophobically bonded β-LG dimer, trimer, etc., generated as a result of the pressure treatment, and were probably aggregates of non-native monomers.

2D SDS:SDS^R-PAGE. The PAGE patterns of the control and the samples that were pressure treated for 20, 60, or 120 min are shown in **Figure 7E**−**H**. Each figure also contains the stained sample gel strip (**a**″) from the 1D SDS-PAGE and the molecular weight standards (Mr). The reduced sample loaded in the left-hand lane (denoted by SR) was the reduced sample of the corresponding sample used in the 1D SDS-PAGE (see **Figure 5C**).

Control Sample. The 2D SDS:SDS^R-PAGE pattern of the control sample is shown in **Figure 7E**. Most of the material shown in the 2D gel is close to the diagonal, indicating that only small changes in mobility had occurred as a consequence of the reduction of the sample. The major spots on the diagonal from the lower right-hand side were as follows: α-LA, β-LG, caseins, IgG$_1$ (light chain), IgG$_1$ (heavy chain), BSA, and LF. A pair of spots to the left of the diagonal appeared to be reduction products that had been dissociated from the protein bands in the sample gel strip. One was identified as IgG$_1$ (light chain), which is known to be separated from IgG$_1$ (heavy chain) under reducing conditions. The other band was identified as monomeric β-LG, which was dissociated from dimeric β-LG in the sample gel strip. A small quantity of disulfide-linked β-LG dimer was found in the control WPC sample. The presence of small quantities of dimeric β-LG in commercial WPCs is common (*50*).

Pressure-Treated Samples. There were also small spots that corresponded to trimer and higher polymers of β-LG in the pressure-treated samples (**Figure 7F−H**). After 20 min of pressure treatment (**Figure 7F**), there were more spots to the left of the diagonal. The most significant spots were in a horizontal line that aligned with the large monomeric β-LG band in the left-hand lane. From right to left in the 2D PAGE pattern, there was a large monomer spot followed by a distinct dimer spot, then a distinguishable trimer spot, and then a band with a large spot corresponding to the band in the $\underline{\mathbf{X}}_4$ region of the sample gel strip (**Figure 5B**). Further to the left was a β-LG streak that corresponded to $\underline{\mathbf{X}}_5$ and $\underline{\mathbf{X}}_6$ of the sample gel strip (**Figure 5B**).

A similar pattern of faint spots and a horizontal line was also observed between the α-LA spot and the α-LA band in the pattern of the reduced sample in the left-hand lane (**Figure 7F−H**). This result suggested that disulfide-bonded dimers, trimers, etc. and complexes of β-LG with α-LA and BSA were formed during the pressure treatment of WPC solutions. The low levels of α-LA polymers compared with β-LG polymers as well as the moderate level of α-LA monomer even after pressure treatment for 120 min at 800 MPa (**Figure 6**) reinforced the notion that α-LA was not as responsive as β-LG to high-pressure treatment.

Consequently, it can be concluded that pressure had generated disulfide-bonded polymers of different whey proteins, primarily from β-LG, that could be dissociated by reduction in the presence of SDS.

DISCUSSION

The 2D native:SDS-PAGE data (**Figure 7A−D**) show that medium- to large-sized polymers can be built up from smaller aggregates such as non-native monomers, dimers, trimers, etc. These results are consistent with the TEM patterns (**Figure 3**), which showed that there was an increase in the larger aggregates, which were apparent after 20 min of pressure treatment (**Figure 3B**). The SEC pattern (**Figure 4B**) also indicates that the native protein peak had diminished significantly after 20 min of pressure treatment. It has been postulated that β-Lg, being a major whey protein, plays a major role in the thermal (*63*, *64*) or pressure-induced (*34*, *35*) aggregation and gelation of WPC. Therefore, the major part of the discussion in the present publication has been focused on the effects of HP on β-LG. However, it will be useful to begin with the knowledge that heated WPC solutions contained 1:1 disulfide-bonded adducts of α-LA and β-LG and that these were more obvious at lower WPC concentrations (*50*). This reaction was further explored by Hong and Creamer (*19*), to show that β-LG could catalyze the formation of α-LA disulfide-bonded dimers and could form α-LA and β-LG adducts. However, analysis of the pressure-treated samples (**Figure 7F−H**) showed that there was very little dimeric material containing α-LA, viz., α-LA dimer, α-LA:BSA, or α-LA:β-LG, but that β-LG dimer was a major intermediate. More of such smaller adducts and intermediate aggregates were observed when WPC solutions were pressure treated at significantly lower concentrations (Patel, H. A.; Singh, H.; Creamer, L. K. Unpublished results, 2005), as was the case for heat-treated samples (*50*); examination of a model system would probably further clarify the situation.

The loss of nativelike or SDS monomeric α-LA (**Figures 5** and **6**) from the pressure-treated WPC solutions was moderate, but this may not be so if α-LA alone is pressure treated (Patel, H. A.; Singh, H.; Creamer, L. K. Unpublished results, 2005); in milk (*14*), α-LA is not affected at pressures of 400 MPa or less. Of course, when a WPC solution is heated, α-LA is lost from the system at almost similar rates to those for β-LG and BSA (*40*, *50*, *65*). The mechanism suggested involvement of thiols, notably CysH121 of β-LG in the first instance (*19*, *57*), catalyzing disulfide bond interchange within a suitable environment (suggested to be a molten globule) involving both α-LA and β-LG (*66*). It is unlikely that the type of environments, such as hydrophobic/molten globule that exist at high temperatures are comparable with those that will be available at very high pressures, in which hydrophobic clusters are less stable (*23*, *28*). However, it has been shown by Hong and Creamer (*19*) that α-LA heat denatures faster in the presence of β-LG, probably because these proteins can form a stable adduct, which can then undergo thiol-catalyzed disulfide bond interchange within the hydrophobic interior of the adduct. This mechanism could apply equally well to the pressure-induced denaturation and aggregation of α-LA in a mixture of whey protein solutions.

The comparison of the present results with the reported literature suggests that there are few similarities between heat- and HP-induced aggregations of whey proteins. The significant differences in the denaturation and aggregation of proteins are mainly related to the rupture of noncovalent interactions and subsequent reformation of intra- and intermolecular bonds by heat as compared with HP (*67*). It is therefore expected and has been demonstrated in various reports that the β-LG gels induced by HP may exhibit different properties from those made by heat treatment. Various studies suggested that the structure and the properties of heat- and pressure-induced gels of β-LG are different (*31*, *41*, *42*). In general, pressure-induced gels are weaker, less elastic, and more exudative than heat-induced gels (*31*) and HP treatment generates gels with more porous structures and lower firmness as compared with heat-induced gels (*31*, *68*, *69*). However, gel formation within the neutral pH range is explained by the build-up of intermolecular disulfide bonds, which connect the whey proteins to form a network structure (*21*, *34*, *43*, *68*, *70*), which is common for both heat- and HP-induced gels. In this context, the results of the present study showed that samples of 12% WPC solutions that were pressure treated at 800 MPa at 22 °C contained a range of disulfide-bonded and hydrophobically bonded protein aggregates after pressure release (**Figure 7**) and gave viscous solutions or gels (**Figures 2** and **3**). Samples that had been pressure treated for longer than 40 min contained particles (e.g., **Figure 3C−F**) that appeared to be comparable with those found by others (*35*, *43*) and were similar to the particulate structure induced by heat treatment (*52*, *71*).

On the basis of earlier studies (*16*, *17*) and as discussed by Considine et al. (*56*), it appears that, at neutral pH, β-LG progressively unfolds as the pressure increases to about 300 MPa. At this pressure, there are only transient structural elements (helices, strands, and sheets) and almost no extensive structure. Disulfide bonds that originally stabilized the native structure of β-LG are strained, and in the presence of a free thiol, inter- and intramolecular disulfide bond interchange occurs readily (*57*). At high pressures, where the hydrophobic effect, which helps to cluster the hydrophobic side chains, is significantly diminished (*67*, *72*), the new disulfide bonds will be different from those in a heat-induced aggregation (*16*, *56*, *57*) and will vary with pressure. At 800 MPa and the times used in the present study, the proteins will have very little classical tertiary structure, but β-LG clearly associates progressively into disulfide-bonded dimers, trimers, etc. (**Figures 5−7**) to give much larger aggregates.

To accommodate these two different viewpoints [viz., more time at high pressure gives more aggregation or gelation with particulate structure, which is observable after depressurization (**Figure 3**), but NMR and other data at higher pressures indicate that stable hydrophobic interactions are unlikely], another process needs to be involved. It is possible that the particles seen in **Figure 3** are not present at 800 MPa but are formed rapidly during (or after) pressure release and that the energy balance that favors hydrophobic interactions at lower pressures encourages the formation of these small particles. This explanation is supported by the larger proportion of protein in the particulate form that occurs between 60 and 90 min of pressure treatment, when the extent of interprotein disulfide bonding is at an intermediate stage (**Figures 5**−**7**). Many assumptions in the reported literature are based on the theory that pressure favors reactions that reduce the volume of the system (*67*, *72*−*74*). In situ, pressure-induced gels are mainly stabilized by hydrogen bonds because this reaction favors a negative reaction volume. Gel formation will be restrained as the pressure increases, if considerable hydrophobic interaction is involved, because the reaction volume is positive (*73*). In such cases, it is assumed that the microstructure of the gel is built up during the pressure release phase because, as the pressure is reduced, hydrophobic interactions are again reinforced and the groups involved reorient themselves. With increasing pressure, protein molecules will undergo a sequence of conformational changes because the balance of stabilizing interactions is altered (*74*). However, during the pressure release phase and after pressure treatment, new intermolecular interactions are formed and the proteins may be newly structured (*75*). Changes in solvation volume are caused mainly by pressure-induced ionization, changes in solvent exposure of amino acid side chains and peptide bonds, and diffusion of water into cavities located in the hydrophobic core of the protein (*67*, *76*, *77*). Such phenomena may be partly supported by other reports (*31*) that indicated that, in contrast to thermal gels, pressure-induced gels of β-LG underwent mechanical and protein solubility changes when stored at 4 °C following pressure release. This clearly indicated a time-dependent strengthening of protein−protein interactions, probably because primary aggregates of β-Lg further aggregated during storage through hydrophobic interactions and disulfide bonds.

As shown in the present study, the proteins in WPC solutions interact and gradually aggregate to form polymers of increasing size with time of pressure treatment at 800 MPa. The identification of dimers, trimers, etc. of β-LG and the other labile whey proteins using 2D SDS:SDS^R-PAGE suggest that disulfide bond interchange is involved, as previously suggested in a number of other studies (*15*, *16*, *43*, *78*). The progress toward gel formation is slow relative to heat-induced gelation, and this must be constrained either by the low rate of thiol-catalyzed disulfide interchange or by the overall low energy gain as a result of gel formation.

Overall, the results of this study on a 12% (w/v) WPC solution at neutral pH are self-consistent and can be interpreted to give a coherent picture of the changes to the protein components and their aggregation that occur during pressure treatment (800 MPa at 22 °C). It is likely that several β-LG molecules will become linked to one another by new disulfide bonds and that there will be a number of CysH residues that are capable of interacting with the disulfide bonds of other molecules. α-LA, as the second major protein, is a potential candidate for forming a disulfide bond with the newly formed β-LG strands and with available CysH residues. At this pressure, there will be negligible β-LG secondary structure, but α-LA will probably have some secondary structure, because of the stability of the molten globule state of this protein (*19*, *23*, *28*). Thus, α-LA will react slowly with the thiols of β-LG and will become incorporated into the growing protein strands and aggregates. It is envisaged that, after about 90 min of pressure treatment, most of the proteins will have become attached to one another via disulfide bonds and that there will be virtually no strong protein−protein interaction or association other than via disulfide bonds. This situation is very different from that in heat-treated WPC solutions, where hydrophobicity appears to be an important driving force. Once the pressure is reduced to atmospheric, the hydrophobic associations will be partly restored within the constraints of the new disulfide bonding structure.

ABBREVIATIONS USED

1D, one-dimensional; 2D, two-dimensional; 2-ME, 2-mercaptoethanol; α-LA, α-lactalbumin; α-LA*, glyco-α-LA; β-LG, β-lactoglobulin; BSA, bovine serum albumin; CD, circular dichroism; HP, high hydrostatic pressure; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; LF, bovine lactoferrin; N′, non-native β-LG; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SDS^R, SDS of reduced samples; SEC, size exclusion chromatography; TEM, transmission electron microscopy; WPC, whey protein concentrate; WPI, whey protein isolate.

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