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# Effects of pressurized thermal processing on native proteins of raw skim milk and its concentrate

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## ABSTRACT

Heating, pressurization, and shearing can modify native milk proteins. The effects of pressurized heating (0.5 vs. 10 MPa at 75 or 95°C) with shearing (1,000 $s^{-1}$) on proteins of raw bovine skim milk (SM, ~9% total solids) and concentrated raw skim milk (CSM, ~22% total solids) was investigated. The effects of evaporative concentration at 55°C and pressurized shearing (10 MPa, 1,000 $s^{-1}$) at 20°C were also examined. Evaporative concentration of SM resulted in destabilization of casein micelles and dissociation of $\alpha_{S1}$- and β-casein, rendering CSM prone to further reactions. Treatment at 10 MPa and 1,000 $s^{-1}$ at 20°C caused substantial dissociation of $\alpha_{S1}$- and β-casein in SM and CSM, with some dissociated caseins forming shear-induced soluble aggregates in CSM. The pressure applied at 10 MPa induced compression of the micelles and their dissociation in SM and CSM at 75 or 95°C, resulting in reduction of the micelle size. However, 10 MPa did not alter the mineral balance or whey proteins denaturation largely, except by reduction of some β-sheets and α-helices, due to heat-induced conformational changes at 75 and 95°C.
**Key words:** pressure, heat, shear, casein micelle

## INTRODUCTION

Heat treatments are commonly applied to raw bovine milk in the dairy industry to achieve microbial safety and to extend shelf life (e.g., pasteurization and sterilization) or as a preparatory step to enhance the functional properties of some dairy products (e.g., cheese and yogurt). Heating of milk, mostly above 70°C, can result in physicochemical changes to proteins, predominantly denaturation and aggregation, depending on temperature and time combination. Unlike caseins, whey proteins are relatively heat-labile. Being the most abundant whey protein, β-LG usually leads heat-induced protein denaturation and subsequent aggregation with other whey proteins, such as α-LA, BSA, immunoglobulins, and lactoferrin (**LF**), as well as the caseins, mainly κ-CN, via thiol, disulfide, electrostatic, and hydrophobic interactions (Patel et al., 2006; Wijayanti et al., 2014; Bogahawaththa et al., 2019).

Concentration of milk by removal of water is an essential step of producing milk powders and other milk concentrates (e.g., evaporated milk and condensed milk), which leads to dissociation of the casein micelles into the serum phase, modification of mineral distribution, conformational changes of whey proteins, and association of β-LG with the casein micelles (Markoska et al., 2019a). These modifications can lead to further destabilization of the casein micelles and conformational changes of β-LG during heating, resulting in compromised heat stability of proteins, depending on the concentration level and combination of temperature and time (Huppertz, 2016; Markoska et al., 2019b). The milk can also be subjected to many mechanical forces, including shear, at various steps during commercial thermal processing (e.g., mixing, stirring, pumping, flowing through pipes, and spraying), which can induce whey protein denaturation, influence protein aggregation, and modify the casein micelles (Mediwaththe et al., 2018a; Bogahawaththa and Vasiljevic, 2020).

Application of high hydrostatic pressure (**HHP**), especially at higher levels (100–1,000 MPa), has been recognized as a promising nonthermal method for pasteurization and sterilization of dairy products and for modification of the functional properties of milk proteins. However, HHP treatments can result in denaturation of whey proteins and their interaction with other proteins to a varying degree, depending on protein type and the combination of pressure and holding time. α-LA is the most pressure-stable (>400 MPa at 40°C for 30 min), and β-LG appears to be the most pressure-sensitive (>100 MPa at 4°C for 30 min; Huppertz et al., 2004b). β-LG tends to form aggregates mainly with κ-CN at ≥600 MPa (Huppertz et al., 2004b; Patel et

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al., 2006; Bogahawaththa et al., 2018). Furthermore, HHP treatments at >200 MPa can modify the casein micelle size. For instance, the micelle size increased (~25%) reversibly at 250 MPa but decreased (~50%) irreversibly at ≥300 MPa in raw skim milk (**SM**; Huppertz et al., 2004b). Pressurization (HHP) of raw SM at the level of 100 to 600 MPa caused the increase of $\alpha_{S1}$- and β-CN in the serum phase due to weakening of hydrophobic interactions and solubilization of colloidal calcium phosphate (**CCP**; Huppertz et al., 2004a).

The application of pressure (>100 MPa) combined with moderate temperature (40–70°C) can modify the physicochemical properties of milk proteins differently than a pressure treatment at ≤30°C. For instance, pressurization at 200 to 800 MPa with 70°C resulted in significantly greater denaturation of β-LG and α-LA and larger particle size in reconstituted SM compared with to the corresponding pressure treatments performed at 20°C (Anema, 2008). When SM was pressurized at 100 to 300 MPa at different temperature levels (25–60°C), the increase in temperature resulted in gradual increase in denaturation of β-LG at the respective pressure levels (López-Fandiño and Olano, 1998b). When raising the temperature from 10 to 40°C at 200 MPa, the particle size of SM increased due to protein aggregation, whereas at ≥400 MPa the degree of disintegration of the casein micelle increased (Anema et al., 2005).

According to the existing literature (Anema, 2008; Huppertz et al., 2019; Nunes and Tavares, 2019; Wijayanti et al., 2019), it appears that no scientific studies have investigated the effects of low pressure (≤10 MPa) combined with heating (>70°C) and shearing on native and concentrated milk proteins. However, such combinations are frequently applied in milk processing, especially heating the milk at >100°C and milk homogenization (55–80°C and 10–25 MPa; Bylund, 2003). For instance, UHT treatment (138–145°C for 3–5 s) is performed under pressurized conditions (~0.4 MPa; Deeth and Datta, 2011). Hence, the current study aimed to examine the effects of pressurized (10 vs. 0.5 MPa) heat treatments (75 or 95°C) with a constant shearing (1,000 $s^{-1}$) on physicochemical and structural changes to native proteins of raw SM and its concentrate. Findings of this work will help in the development or optimization of thermal processing parameters to achieve desired product characteristics.

## MATERIALS AND METHODS

### Sample Preparation

Murray Goulburn Cooperative Co. Ltd. (Laverton North, VIC, Australia) kindly provided raw bovine milk. Upon delivery, the milk was skimmed by centrifugation, and sodium azide at 0.01% (wt/wt) was added to control potential microbial activities (Markoska et al., 2019a). The standard oven drying method (105°C) was used to determine TS content of the resultant SM, which was 9 ± 0.3% (wt/wt). The SM was then divided into 2 portions, one of which was concentrated by evaporation at 55°C for about 100 min using an R-100 rotary evaporator (John Morris Scientific, Deepdene, VIC, Australia) as described previously (Markoska et al., 2019a) to obtain skim milk with 22 ± 0.2% (wt/wt) TS, which was termed concentrated skim milk (**CSM**).

### Sample Treatment

Both SM and CSM samples were heated to 2 temperature levels (75 or 95°C) at a ~5°C/min heating and cooling rate under 2 low-pressure conditions (0.5 or 10 MPa) using a cup-and-bob geometry (CC 25/PR-SN; Anton Paar, Ostfildern, Germany) placed in a pressure cell (CC25/PR-150; Anton Paar) mounted on a Physica MCR 301 rheometer (Anton Paar), as explained previously (Mediwaththe et al., 2018a). The required pressure levels were generated and maintained using a compressed air system linked to the pressure cell and monitored by an associated software (Rheoplus, Anton Paar). A constant shear at 1,000 $s^{-1}$ was applied with all the treatments, and the cooling process was terminated at room temperature (~20°C). Another aliquot of SM and CSM was pressurized to 10 MPa at 20°C with constant shearing, using the same system, to examine the combined effects of pressure and shearing. Total treatment times (heating and cooling or holding) at 95, 75, and 20°C were ~30, 22, and 20 min, respectively. Untreated samples of SM and CSM at ~20°C were considered the controls. After the treatments, an aliquot of all the treated and the control samples was ultra-centrifuged at 100,000 × *g* for 1 h at 20°C using a Beckman Ultra L-70 (Beckman Coulter Pty Ltd., Lane Cove West, NSW, Australia) to separate the supernatant (serum phase) from the pellet, keeping the other aliquot as the intact milk (termed "bulk milk") before analysis.

### Particle Size and Zeta Potential Measurement

Average particle size and zeta potential of all the treated and control milk samples (bulk milk) was measured at 20°C using a Nano-ZS Zetasizer (Malvern Instruments, Malvern, UK) after diluting them in a simulated milk ultrafiltrate as reported previously (Markoska et al., 2019a). Refractive indices of the casein micelle in milk and the simulated milk ultrafiltrate were set at 1.57 and 1.34, respectively.

### Mineral Content Determination

Calcium, magnesium, and phosphorus content in all the treated and control milk samples and their serum phases were determined using an ICP Multitype inductively coupled plasma atomic emission spectrometer (Shimadzu Corporation, Kyoto, Japan) as described previously (Markoska et al., 2019a). All the samples were ashed and then dissolved in 1 *M* nitric acid before determining the mineral content.

### Polyacrylamide Gel Electrophoresis

The SDS-PAGE analysis was performed under nonreducing and reducing (using β-mercaptoethanol) conditions for all the treated and control milk samples and their serum phase following the method explained previously (Bogahawaththa et al., 2017). Gels containing 30% acrylamide and 10% SDS were used for electrophoresis, and protein bands were stained with Coomassie Brilliant Blue (Sigma-Aldrich Pty Ltd., Castle Hill, NSW, Australia). The gel images were captured using the ChemiDoc Imaging System (Bio-Rad Laboratories, Gladesville, NSW, Australia).

### Fourier-Transform Infrared Spectroscopy

All the milk samples (treated and control) were scanned using a Frontier Fourier-transform infrared spectrometer (PerkinElmer, Waltham, MA). Every spectrum was an average of 16 scans at 4 $cm^{-1}$ resolution in absorbance mode with background (water) subtraction. Changes of secondary structure of the proteins were analyzed using second derivative form of all the spectra within broad amide I region (1700–1,600 $cm^{-1}$) by Spectragryph software (v.1.2.7; Bogahawaththa et al., 2017).

### Statistical Analysis

The entire experiment was replicated with raw milk obtained on 2 different occasions ($n \geq 6$). Data were analyzed by ANOVA and Tukey test, considering the level of significance at $P \leq 0.05$, using SAS statistical software (v. 9.2; SAS Institute Inc., Cary, NC).

## RESULTS AND DISCUSSION

### Effect of Evaporative Concentration on Milk Proteins

To investigate the effects of the evaporative concentration process on milk proteins, a comparison was made between the control (untreated) sample of SM (~9% wt/wt of TS) and the control (without further processing) sample of CSM (~22% wt/wt of TS). Particle size of the control CSM significantly increased (Table 1) compared with the control SM. This can be mainly related to dense packing of the casein micelles during the evaporative concentration and subsequently enhanced protein interactions, especially association of β-LG with the casein micelles (Markoska et al., 2019a). The temperature (55°C) applied during the evaporative concentration was higher than that (30°C) required to dissociate β-LG dimers into monomers and thereby expose hydrophobic sites, leading to increased protein interactions (Wijayanti et al., 2014; Markoska et al., 2019a). Furthermore, a substantial release of $\alpha_{S1}$- and β-CN into the serum phase, as observed in the nonreducing SDS-PAGE results (Figure 1, B1 and D1), indicated a considerable dissociation of the casein micelles during the concentration step. This can be attributed to solubilization of CCP as well as to weakening of electrostatic interactions and hydrogen bonding, leading to destabilization of the casein micelles (Markoska et al., 2019a). Anema and Klostermeyer (1997) reported that ~7% of β-CN and ~5% of $\alpha_S$-CN were in the serum phase of reconstituted SM (10% wt/wt of TS) after heating at 50 to 60°C for 15 min, and this temperature level had the greatest effect on dissociation of the caseins into the serum phase within the 20 to 100°C range. Hence, the 55°C applied during the concentration phase also contributed to the dissociation of $\alpha_{S1}$- and β-CN in the current study. Markoska et al. (2019a) reported similar results to the current study: for instance, increase in size of the casein micelle, increase in the soluble caseins in the serum phase, and related destabilization of the casein micelles when raw SM (9% wt/wt of TS) was evaporatively concentrated into 17 or 25% wt/wt of TS using the equivalent conditions.

Some structural changes of the protein secondary structures were also observed via Fourier-transform infrared spectrometry (Figure 2) upon concentration. For instance, a slight increase in antiparallel β-sheets (~1638–1,632 $cm^{-1}$), aggregated β-sheets (~1690–1,685 $cm^{-1}$), and α-helices (~1653–1,651 $cm^{-1}$) (Bogahawaththa et al., 2019; Markoska et al., 2019a) was observed in the control CSM compared with the control SM (Figure 2A). These changes can be related to increased protein concentration and, thereby, close molecular packing (Markoska et al., 2019a). We detected no significant changes of the final mineral balance when the soluble minerals of SM (Table 2: Ca 12.1, Mg 3.6, and P 13.6 m*M*) were compared with concentration-normalized soluble minerals of CSM (Ca 13.5, Mg 3.4, and P 14.5 m*M*). A slight increase in Ca and P in the serum phase of the control CSM (concentration normalized), compared with the control SM, indicated solubilization of CCP to some extent, which was, however, not promi-

nent, due to movement of Ca and P into the micellar phase during the concentration phase (Nieuwenhuijse et al., 1988). The zeta potential did not change ($P > 0.05$) after the concentration step (Table 1), as reported previously (Markoska et al., 2019a).

### Influence of Pressurized Shearing at 20°C on Milk Proteins

Any changes of the proteins that can be observed between the control (~20°C) and samples of SM or CSM treated at 10 MPa at 20°C with 1,000 $s^{-1}$ can be related to the combined effect of pressure (10 MPa) and shearing (1,000 $s^{-1}$). We detected no significant change (only a slight increase) of the particle size of SM or CSM following the 10-MPa, 20°C treatment (Table 1). From the SDS-PAGE nonreducing image (Figure 1, B1), slightly intense $\alpha_{S1}$- and β-CN bands appeared in the serum phase of the SM treated at 10 MPa and 20°C compared with those of the control, whereas the whey protein bands did not change. This indicated that 10 MPa and 20°C under shear treatment contributed to destabilization of the casein micelles slightly, which resulted in dissociation of the caseins into the serum phase without altering the micelle size considerably. Because the low pressure applied (10 MPa) at 20°C is unlikely to change the micellar structure (Huppertz et al., 2004a; Anema et al., 2005), destabilization of the casein micelles can be ascribed to the applied shear or the combination of shear and pressure. Reversible destabilization of the casein micelle, leading to increase in its size, was reported when raw SM was sheared at 1,000 $s^{-1}$ and 20°C in an equivalent experimental setting, due to fluid grads created by shearing in the flow direction (Mediwaththe et al., 2018a).

In contrast, very faint $\alpha_{S1}$- and β-CN bands were observed from the serum phase of CSM treated at 10 MPa and 20°C under nonreducing SDS-PAGE (Figure 1, D1) compared with those of the control. The intensity of these bands, however, was mostly similar under reducing SDS-PAGE conditions (Figure 1, D2). This suggested that the dissociated caseins were involved in formation of soluble aggregates potentially induced by shearing. Apart from the caseins, it appeared that some whey proteins, especially minor whey proteins (IgG, LF, and BSA), were also involved in this aggregation process, because they displayed relatively more intense bands, as indicated by the reducing SDS-PAGE of the CSM treated at 10 MPa and 20°C, compared with that of the control (Figure 1, D2). However, the participation of β-LG and α-LA in the protein aggregation was not obvious under these treatment conditions. The denser molecular packing, particularly of larger molecules such as minor whey proteins (IgG, LF, and BSA), and deterioration of protein stability take place during the concentration step (Markoska et al., 2019a). In addition to this, shear-induced structural modifications (Mediwaththe et al., 2018a,b; Bogahawaththa and Vasiljevic, 2020) can create an environment to induce inter- and intraprotein interactions, leading to their aggregation. Pressurization and shearing at 20°C did not change the secondary structure of the proteins in SM or CSM substantially, probably due to least physicochemical changes in major whey proteins (β-LG and α-LA), as discussed above. However, some reduction of the antiparallel β-sheets and α-helices was observed that can be related to the dissociation of β-LG dimers into monomeric form (Lefèvre and Subirade, 1999) and partial unfolding of the native conformation of β-LG induced by shearing (Mediwaththe et al., 2018a). The soluble Ca, Mg, and P in SM and CSM largely decreased following the pressurized shear treatment, compared with those of the control, possibly due to shifting of the mineral balance induced by shear. Similar to the

**Table 1.** The average particle size and zeta potential of skim milk and concentrated skim milk samples subjected to different pressurized thermal treatments

| Sample | Temperature (°C) | Pressure (MPa) | Particle size (nm) | Zeta potential (mV) |
|---|---|---|---|---|
| Skim milk (~9% wt/wt TS) | Control (~20) | NA[1] | $178.9 \pm 1.7^{de}$ | $-18.3 \pm 1.0^{ab}$ |
| | 20 | 10 | $179.7 \pm 2.6^{de}$ | $-17.7 \pm 0.3^{ab}$ |
| | 75 | 0.5 | $177.3 \pm 1.9^{e}$ | $-17.9 \pm 2.1^{ab}$ |
| | 75 | 10 | $173.3 \pm 2.2^{e}$ | $-18.8 \pm 0.8^{b}$ |
| | 95 | 0.5 | $191.2 \pm 4.1^{ab}$ | $-18.4 \pm 0.5^{ab}$ |
| | 95 | 10 | $184.6 \pm 1.4^{cd}$ | $-16.4 \pm 0.7^{ab}$ |
| Concentrated skim milk (~22% wt/wt TS) | Control (~20) | NA | $186.6 \pm 2.3^{bc}$ | $-17.8 \pm 1.9^{ab}$ |
| | 20 | 10 | $187.9 \pm 5.8^{bc}$ | $-17.0 \pm 0.7^{ab}$ |
| | 75 | 0.5 | $188.1 \pm 1.5^{bc}$ | $-16.9 \pm 0.5^{ab}$ |
| | 75 | 10 | $179.2 \pm 1.6^{de}$ | $-17.3 \pm 1.1^{ab}$ |
| | 95 | 0.5 | $195.9 \pm 1.2^{a}$ | $-17.0 \pm 0.5^{ab}$ |
| | 95 | 10 | $190.1 \pm 1.5^{abc}$ | $-15.9 \pm 1.6^{a}$ |

[a–e]Mean values without a common superscript letter in the same column indicate significant difference ($P \leq 0.05$).

[1]NA = not applicable.

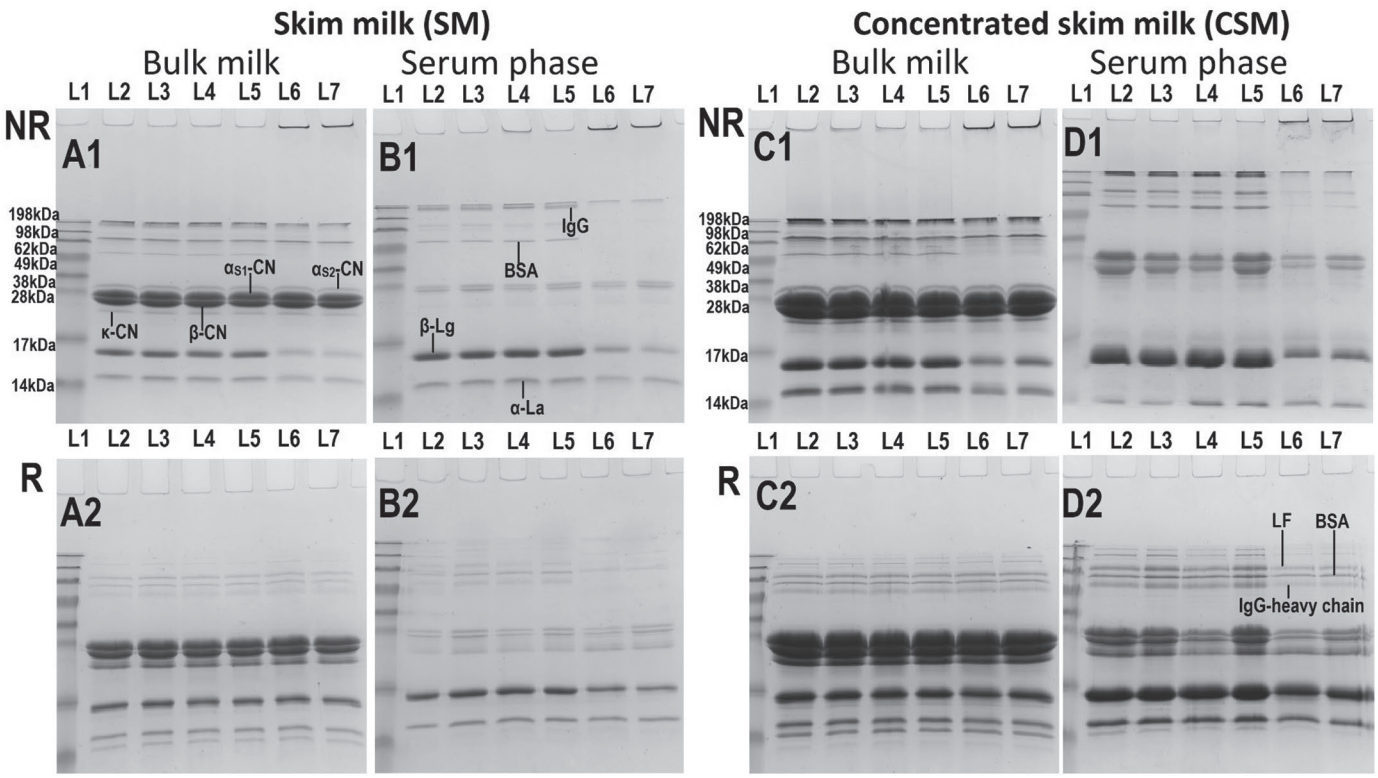


**Figure 1.** Sodium dodecyl sulfate-PAGE images of skim milk (SM) and concentrated skim milk (CSM) samples (bulk milk) subjected to different pressurized thermal treatments and their supernatants (serum phase) obtained via ultracentrifugation after treatments. A1 and C1 are the SM and CSM bulk samples, and B1 and D1 are their serum phases, respectively, under nonreducing (NR) conditions. A2, B2, C2, and D2 are the corresponding reducing (R) images. Lanes (L) are as follows: L1 = molecular weight marker, L2 = control, L3 = 10 MPa at 20°C, L4 = 0.5 MPa at 75°C, L5 = 10 MPa at 75°C, L6 = 0.5 MPa at 95°C, and L7 = 10 MPa at 95°C. Protein bands are β-LG, α-LA, IgG, lactoferrin (LF), BSA, $\alpha_{S1}$-CN, $\alpha_{S2}$-CN, β-CN, and κ-CN.

concentration step, the zeta potential of SM or CSM did not change significantly ($P > 0.05$) after this treatment.

### *Effects of Pressurized Thermal Processing at 75 and 95°C on Milk Proteins*

Examination of the effects of low pressure (10 MPa) on milk proteins at 75 or 95°C is the main focus of this section, and effect of temperature was considered when relevant. Thus, 10 MPa was compared with 0.5 MPa (0.5 MPa was considered the base pressure level) at each temperature level separately (75 or 95°C), where constant shearing (1,000 $s^{-1}$) was also applied in all treatments. The average particle size of SM was slightly reduced after treatment at 10 MPa and 75°C compared with 0.5 MPa at 75°C. On the other hand, the particle size significantly decreased in CSM subjected to 10 MPa at 75°C compared with that treated at 0.5 MPa and 75°C (Table 1). A mostly similar trend was observed in both SM and CSM at 95°C, as the particle size of the samples treated at 10 MPa was reduced more than those subjected to 0.5 MPa pressure. Pressurization can substantially reduce casein micelle size due to compression, as observed in reconstituted SM after high-pressure and low-temperature treatments (100–200 MPa, 20°C, 20 min; Anema et al., 2005). Furthermore, increase in treatment temperature from 10 to 40°C at 100 MPa exhibited a decreasing trend of micelle size (Anema et al., 2005). The micelle size of SM significantly increased when a range of high-pressure treatments (200–800 MPa) were applied at 70°C for 30 min, compared with their counterparts at 20°C, using a high-pressure processor equipped with a water jacket (Anema, 2008), potentially due to association of the denatured β-LG with the casein micelles (Huppertz et al., 2004b) or to the formation of large protein aggregates due to various interactions between caseins and whey proteins (Bogahawaththa et al., 2018). Hence, the observations in the current study suggest that the low-pressure, high-temperature treatments (10 MPa, ≥5°C) had effects on casein micelles similar to those

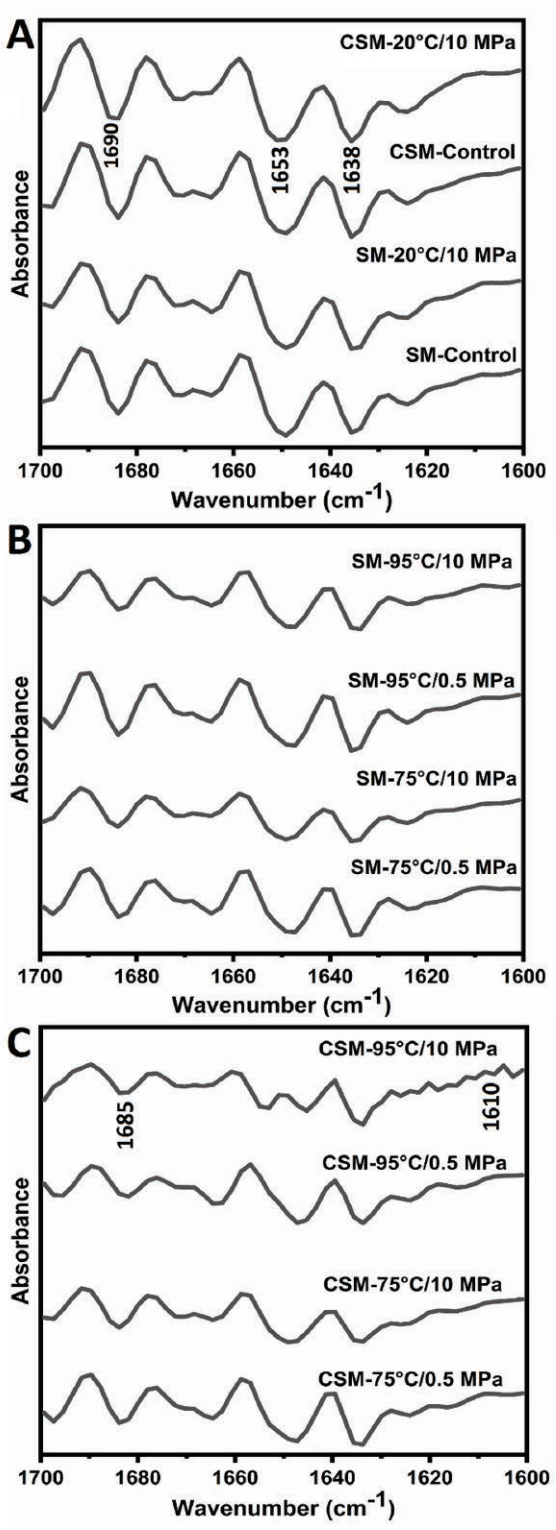


**Figure 2.** Second derivative of the Fourier-transform infrared spectra obtained from skim milk (SM) and concentrated skim milk (CSM) samples subjected to the different pressurized thermal treatments and their untreated controls (Control). (A) Control and samples of SM and CSM treated at 20°C and 10 MPa; (B) SM samples treated at 0.5 or 10 MPa and at 75 or 95°C; (C) CSM samples treated at 0.5 or 10 MPa and at 75 or 95°C.

of high-pressure, low-temperature treatment (100 MPa, 20°C, ~20 min) applied using HHP systems (Anema et al., 2005; Anema, 2008).

When comparing the protein bands between SM treated with 0.5 MPa at 75°C, versus 10 MPa at 75°C, and their serum phases, no substantial changes were observed for whey proteins in the nonreducing SDS-PAGE images (Figure 1, A1, B1). However, we observed relatively intense $\alpha_{S1}$- and β-CN bands from the serum phase of SM treated with 10 MPa at 75°C, compared with those treated with 0.5 MPa at 75°C (Figure 1, B1). A similar trend of results was observed between SM treated with 0.5 MPa at 95°C and 10 MPa at 95°C and their serum phases under nonreducing SDS-PAGE conditions. These intense casein bands observed at 10 MPa, compared with 0.5 MPa, at 75 or 95°C, can be related to further pressure-induced destabilization of the casein micelles and dissociation of the caseins due to solubilization of CCP and interruption of hydrogen bonds (Patel et al., 2006), which were also affected by heating (Anema, 1998). This observation appears to accord with an effect of the high-pressure, low-temperature treatments (≥100 MPa, 20°C, ~20 min) on the casein micelles (López-Fandiño et al., 1998a; Huppertz et al., 2004a; Anema et al., 2005).

Furthermore, no apparent changes in the $\alpha_{S1}$- and β-CN bands were noticed between 75 and 95°C at each pressure level (0.5 or 10 MPa) in SM, indicating heat stability of the micellar structure (Anema, 1998). However, the whey proteins in SM increasingly denatured at 95°C, displaying substantially faint bands of β-LG and α-LA as well as disappearance of BSA and IgG bands compared with those treated at 75°C (Bogahawaththa and Vasiljevic, 2020), without obvious pressure dependence, confirming their high heat-lability (Wijayanti et al., 2014, 2019). It also appeared that these whey proteins formed aggregates with the involvement of κ-CN at 95°C, as seen on the stacking gel of the nonreducing SDS-PAGE (Figure 1, B1), which disappeared in the reducing gels (Figure 1, B2) due to reduction of the covalent bonds by β-mercaptoethanol. This confirmed that the protein aggregates were primarily formed by thiol (disulfide) bonds, as reported previously (Bogahawaththa and Vasiljevic, 2020). The formation of protein aggregates was also indicated by the substantially increased particle size in the SM samples treated at 95°C compared with those treated at 75°C, and appeared to be pressure-dependent. The whey protein bands of CSM and its serum phase did not display substantial changes between 0.5 and 10 MPa treatments at 75 or 95°C, as indicated by the nonreducing SDS-PAGE images (Figure 1, C1 and D1).

The $\alpha_{S1}$- and β-CN bands of the serum phase of CSM treated with 10 MPa were more intense than those of

the samples treated with 0.5 MPa at 75 or 95°C (Figure 1, D1). Similar results were observed in the corresponding SM samples (with relatively faint bands due to the low protein concentration) and can be discussed in the same way in relation to the pressure-induced destabilization of the casein micelles. This also agrees with the substantially smaller average particle size of CSM treated at 10 MPa compared with that treated at 0.5 MPa at 75 or 95°C. However, the $\alpha_{S1}$- and β-CN bands of the serum phase of CSM treated at 75°C were prominent, whereas those treated at 95°C were relatively faint depending on the applied pressure. These casein bands in the serum phase of SM appeared to change depending only on the pressure (0.5 MPa vs. 10 MPa) but not the temperature levels (75 and 95°C). Markoska et al. (2019b) reported no changes in the $\alpha_s$- and β-CN bands of the serum phase when raw SM (9% wt/wt TS) was heated from 75 to 110°C, whereas intensity of these casein bands gradually reduced in the serum phase of the CSM (17 or 25% wt/wt TS) during the same heating ramp. This gradual reduction of $\alpha_s$- and β-CN bands in the serum phase of the CSM was ascribed to their reassociation with the casein micelles, which largely dissociated (reversibly) at 75°C. Anema (1998) reported that when reconstituted CSM (17.5 or 25% wt/wt TS) was heated from 20 to 120°C, the maximum dissociation of the casein micelles was observed at 60 to 80°C, which resulted in the highest content of soluble $\alpha_s$- and β-CN in the serum phase. However, κ-CN progressively dissociated from the casein micelles with increase in temperature from 75 to 110°C in raw SM (Markoska et al., 2019b) and from 20 to 120°C in reconstituted SM (Anema, 1998) regardless of their TS (unconcentrated or concentrated), and formed soluble and insoluble aggregates with the involvement of the whey proteins, predominantly β-LG and α-LA (Markoska et al., 2019b). A similar behavior was demonstrated by κ-CN in the current study. The κ-CN band in the serum phase of SM or CSM treated at 95°C was more intense, due to disintegration of thiol (disulfide)-linked κ-CN and whey-protein soluble aggregates, than those treated at 75°C, depending on the pressure applied (Figure 1, B2, D2).

The Fourier-transform infrared spectrometry results of SM and CSM demonstrated substantial reduction of β-sheet and α-helix structural elements in the samples treated at 10 MPa compared with the 0.5 MPa treatment at 75 or 95°C (Figure 2, B, C). This could be ascribed mainly to loss of native confirmation of the whey proteins, as observed previously, after the high-pressure, low-temperature treatments (≥100 MPa, 20–30°C, ~20 min; Maresca et al., 2017; Bogahawaththa et al., 2018). Treatment of CSM with 10 MPa at 95°C resulted in a relatively greater loss of α-helices and appearance of a few new peaks in the β-sheet region (~1638–1,610 $cm^{-1}$; Bogahawaththa et al., 2019), displaying some molecular rearrangements. We found no major changes ($P > 0.05$) in zeta potential between SM or CSM treated with 0.5 MPa and 10 MPa at 75 or 95°C. However, SM and CSM had slightly less negative surface potential after treatment at 10 MPa and 95°C compared with those of the control, indicating protein aggregation mainly induced by elevated temperature and shearing (Mediwaththe et al., 2018b), as observed from the nonreducing SDS-PAGE.

The pressure applied (10 MPa) did not modify the mineral balance of SM or CSM significantly at 75 or 95°C (except Mg in SM at 95°C and P in CSM at

**Table 2.** The total and soluble mineral concentration of skim milk and concentrated skim milk samples subjected to different pressurized thermal treatments

| Sample | Temperature (°C) | Pressure (MPa) | | Mineral concentration (m*M*) | | |
|---|---|---|---|---|---|---|
| | | | | Ca | Mg | P |
| Skim milk | Control (~20) | NA[1] | Total minerals | 35.2 ± 2.2 | 5.2 ± 0.2 | 30.5 ± 1.4 |
| (~9% wt/wt total solids) | Control (~20) | NA | Soluble minerals | 12.1 ± 0.2$^{e}$ | 3.6 ± 0.1$^{a}$ | 13.6 ± 0.3$^{e}$ |
| | 20 | 10 | | 10.6 ± 0.1$^{ef}$ | 3.0 ± 0.0$^{bc}$ | 11.8 ± 0.0$^{f}$ |
| | 75 | 0.5 | | 10.0 ± 0.3$^{f}$ | 3.1 ± 0.0$^{b}$ | 11.7 ± 0.3$^{f}$ |
| | 75 | 10 | | 10.1 ± 0.1$^{f}$ | 2.9 ± 0.1$^{bc}$ | 11.6 ± 0.1$^{f}$ |
| | 95 | 0.5 | | 10.0 ± 0.5$^{f}$ | 3.4 ± 0.2$^{a}$ | 12.0 ± 0.5$^{f}$ |
| | 95 | 10 | | 9.2 ± 0.1$^{f}$ | 2.8 ± 0.0$^{c}$ | 10.9 ± 0.2$^{f}$ |
| Concentrated skim milk | Control (~20) | NA | Total minerals | 81.8 ± 1.6 | 12.3 ± 0.2 | 72.6 ± 1.8 |
| (~22% wt/wt total solids) | Control (~20) | NA | Soluble minerals | 33.1 ± 1.1$^{a}$ | 8.3 ± 0.1$^{ab}$ | 35.4 ± 0.8$^{a}$ |
| | 20 | 10 | | 29.5 ± 0.8$^{b}$ | 8.1 ± 0.1$^{bc}$ | 32.4 ± 0.7$^{b}$ |
| | 75 | 0.5 | | 30.5 ± 0.9$^{b}$ | 8.4 ± 0.3$^{ab}$ | 33.3 ± 0.4$^{b}$ |
| | 75 | 10 | | 30.3 ± 0.6$^{b}$ | 8.4 ± 0.0$^{a}$ | 35.2 ± 0.4$^{a}$ |
| | 95 | 0.5 | | 22.9 ± 1.2$^{c}$ | 7.9 ± 0.2$^{c}$ | 28.8 ± 0.7$^{c}$ |
| | 95 | 10 | | 21.8 ± 0.7$^{c}$ | 7.5 ± 0.1$^{c}$ | 29.1 ± 0.3$^{c}$ |

[a–f]The mean values of the same soluble mineral without a common superscript letter indicate significant difference ($P \leq 0.05$).
[1]NA = not applicable.

75°C), compared with that at 0.5 MPa, although the casein micelle was substantially altered in the current study in a similar way as if it were subjected to a high-pressure, low-temperature treatment (≥100 MPa, 20°C, ~20 min). High-pressure treatments at ≥100 MPa and 20°C for 15 to 30 min resulted in an increase in soluble minerals (Ca, P, and Mg) in the serum phase of SM (López-Fandiño et al., 1998a) and reconstituted milk protein concentrates (Cadesky et al., 2017), due to disintegration of the casein micelles and movement of Ca, P, and Mg into the serum phase. In contrast, heat treatment can result in movement of the mineral balance into the micellar phase mainly by association of Ca and P with the casein micelles (Gaucheron, 2005). We observed a substantial reduction of Ca, P, and Mg in the serum phase of SM and CSM with increase in treatment temperature from 20 to 95°C, regardless of the pressure applied; this effect was greater in CSM than in SM, as observed previously (Markoska et al., 2019b). Hence, the effect of low pressure (10 MPa) on the mineral balance in SM and CSM appeared to be mostly counterbalanced by the effect of temperature (75 or 95°C) in the current study.

The heat and pressure treatments govern denaturation of the whey proteins differently in the milk. The heat-induced denaturation of whey proteins occurs following increased hydrophobic interactions and reduction of soluble Ca and P in the serum phase. On the contrary, the pressure treatments induce denaturation of the whey proteins through diminished hydrophobic interactions and increased solubility of Ca and P (Anema, 2008). Because the temperature levels were more severe than the pressure applied in the current study, in terms of denaturation of whey proteins (Huppertz et al., 2004b; Patel et al., 2006), the influence of low pressure (10 MPa) on denaturation of whey proteins appeared to be offset by the effect of the high temperature (75 or 95°C). Hence, in the current study, we observed that the increase in temperature from 20 to 95°C accelerated the denaturation of β-LG, α-LA, IgG, LF, and BSA, whereas we detected no significant effect of 10 MPa on denaturation of these whey proteins at 75 or 95°C, as previously discussed.

## CONCLUSIONS

All the variables tested had individual or combined effects on native milk proteins. Evaporative concentration resulted in the destabilization of the casein micelles and dissociation of $\alpha_{S1}$- and β-casein into the serum phase. Generally, CSM appeared to be more prone to further modifications than SM. Pressurized shearing at 20°C contributed to formation of soluble aggregates in CSM, driven mainly by dissociated caseins and minor whey proteins. Temperatures of 75 and 95°C influenced the caseins, whey proteins, and mineral balance, whereas low pressure (10 MPa) appeared to influence the micellar structure regardless of temperature. Treatment of 10 MPa at 75 or 95°C can result in dissociation of casein micelles and reduction of their size in both SM and CSM, mostly in a similar way to that found in a high-pressure, low-temperature treatment (≥100 MPa, 20°C, ~20 min). However, the applied pressure did not greatly modify the mineral balance or accelerate denaturation of whey proteins, due to a counteref-fect exerted by heating. The pressurization contributed to substantial loss of the secondary structure during shearing at 20°C and heating at 75 or 95°C. These results need to be considered for optimum processing of dairy systems.

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