Comparison of the Effects of High Pressure and Thermal Treatments on the Casein Micelles in Goat's Milk

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The influence of thermal and high-pressure treatments on caprine casein micellar structure and, in particular, on the micellar serum distribution of whey proteins, caseins, and calcium phosphate was studied by ultracentrifugation and transmission electron microscopy. Quantification of the major whey proteins and caseins was achieved using reverse-phase HPLC, and the extent of denaturation of the whey proteins was determined by gel permeation FPLC. Thermal treatment of goat's milk had little effect on the micellar serum distribution of caseins, but pressure treatment at 20 °C caused disruption of micelles into smaller particles and gave an increase in the amount of serum casein. Pressure treatments between 300 and 350 MPa at 45 °C caused the formation of very large micelles and gave an increase in the level of serum κ-casein. Higher pressure at 45 °C, however, caused disruption of the micelles, with the formation of smaller, less easily sedimented fragments. On pressure treatment, βLg was most easily denatured, and appreciable denaturation of the immunoglobulins and α-lactalbumin only occurred at the higher pressures, particularly at 45 °C.

Keywords: *Caseins; micelle; high pressure; goat's milk*

INTRODUCTION

Goat's milk has traditionally been used in the manufacture of dairy products, such as cheese and yogurt, for which potentially there is a significant market within the European Union, especially as a result of the introduction of quotas restricting the supply of cow milk. The use of raw goat's milk for cheese-making was formerly widespread, but thermal treatments have been introduced to ensure the hygienic quality (Gay et al., 1993) and in particular to prevent problems caused by contamination with *Brucella* spp. Pasteurization, however, in addition to causing inactivation of vegetative pathogenic microorganisms also results in changes in the milk components and in the characteristics of the curds and cheeses.

Novel methods of food processing have been developed in recent years. Treatments using high isostatic pressure and dielectric pulses inactivate the vegetative forms of most microorganisms without affecting the nutritive value of the food to the same extent as heating. In cow's milk, pressure treatment causes inactivation of microorganisms (Gervilla et al., 1996), enhances rennet coagulation (Desobry-Banon et al., 1994; López-Fandiño et al., 1996), and markedly improves acid coagulation and acid-set gel properties (Johnston et al., 1993; Desobry-Banon et al., 1994). These findings suggest that high-pressure processing could be useful in the production of high quality cheese and yogurt.

Although the effect of different processing treatments on goat's milk has been investigated (Montilla and Calvo, 1997; Villamiel et al., 1997), little information is available about changes in caprine casein micellar

structure. Richardson et al. (1974) reported differences between cow milk and goat milk with respect to micellar size and micellar mineralization. Aoki et al. (1993) similarly found higher levels of caseins cross-linked by micellar calcium phosphate in goat's milk as compared with cow's milk. These differences in micellar structure are reflected in the markedly lower ethanol stability of goat's milk (Horne and Parker, 1982), the severe precipitation that occurs when the milk is subjected to UHT processing (Zadow et al., 1994), and the absence of a reduction in rennet clotting time after heat treatments up to 85 °C for 30 min (Montilla et al., 1995).

Little work has been done on the effects of pressure on goat's milk. In a previous paper, we reported the effects of high pressure and heat treatments on the denaturation of whey proteins in goat's milk (Felipe et al., 1997). In the present work, we have compared the effects of pressure and thermal treatments on casein micellar structure and in particular on the micellar serum distribution of whey proteins, caseins, and calcium phosphate. We have also examined the effects of these treatments on the appearance of caprine micelles as determined by transmission electron microscopy.

MATERIALS AND METHODS

Milk Samples. Fresh, nonrefrigerated milk from Murciano-Granadina goats was obtained from the farm of the Universitat Autònoma de Barcelona. The milk was skimmed to 0.1% fat, and the pH was adjusted, if necessary, to 6.7. Each milk sample for pressure treatment was placed in a screw-topped plastic bottle, vacuum packed, and thermally sealed before treatment.

Heat Treatment. Milk samples (5.0 mL) were heated in stoppered, thin-walled glass tubes (1 mm wall, 8 mm internal diameter, 160 mm length) in a water bath. The tubes were

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allowed to incubate for between 2 min 30 s to 3 min 50 s in order to warm to 70, 75, 80, 85, and 90 °C, were maintained at the selected temperature for 10 min, and then were rapidly cooled in ice.

High-Pressure Treatment. Milk samples were treated in hydrostatic high-pressure equipment (ACB, Nantes, France). Pressure reached 500 MPa in about 4 min, and it took between 90 and 120 s for pressure reduction. Treatment temperature was taken as the water temperature inside the cylinder, measured just before application of pressure. The pressure chamber and the water inside were cooled or heated to the required temperature by a constant flow of fluid within the walls of the vessel. Ten minutes before treatment, milk samples were equilibrated to the desired temperature. Samples were pressurized at 20 or 45 °C, with a holding time of 10 min at the required temperature. When the treatment was complete, some samples were centrifuged immediately, and others were refrigerated for 17 h before analysis.

Ultracentrifugation. Milk samples were centrifuged at 20 °C and 7000g for 30 min, 20000g for 30 min, and 50000g for 120 min, consecutively. After each centrifugation, a sample of the supernatant was removed for analysis. During the final centrifugation, an opalescent layer just above the pellet was observed, and this was carefully removed for later analysis.

Acid Filtrate. Milk samples were warmed to 20 °C and adjusted to pH 4.6 by mixing with an equal volume of buffer consisting of acetic acid (0.83 M) and sodium acetate (0.2 M). After allowing the samples to stand for 15 min, the supernatants were passed through 0.2 μm nylon filters (Sartorius, Epson, Surrey U.K.).

Gel Permeation FPLC. The extent of irreversible denaturation of the main whey protein fractions [immunoglobulins (Ig), serum albumin and lactoferrin (SA/Lf), β-lactoglobulin (βLg), and α-lactalbumin (αLa)] in raw or processed samples was determined by following their loss of solubility at pH 4.6, the individual components in the acid filtrates being separated on a Superdex 75 HR 10/30 column (Law et al., 1993).

Ion-Exchange FPLC. Micellar caseins and associated denatured whey proteins were alkylated or reduced with 2-mercaptoethanol and subsequently fractionated using both anion-exchange FPLC (Law and Tziboula, 1993) on a Mono Q HR 5/5 column and cation-exchange FPLC (Law and Tziboula, 1992) on a Mono S HR 5/5 column (Pharmacia Biotech, St. Albans, U.K.).

Reverse-Phase HPLC Separation of Caprine Proteins. A modification of the method of Visser et al. (1991) was used to separate and quantify the major caseins and whey proteins in milk and supernatants. Proteins were separated on an Apex WP ODS reverse-phase column (7 μm; 4.6 × 250 mm; Jones Chromatography Ltd., Mid-Glamorgan, U.K.) using a gradient of acetonitrile in 0.1% TFA. The acetonitrile gradient was 33−44% in 15 min followed by 44−49% over a further 15 min. Flow rate was 1 mL min^{-1}, column temperature was 46 °C, and detection was at 214 nm.

Calcium and Inorganic Phosphate Analyses. The concentrations of calcium (Ca) and inorganic phosphate (P$_i$) were determined in the supernatants obtained after ultracentrifugation. Protein was precipitated from each of the samples by the addition of 1 mL of 24% w/v trichloroacetic acid to 1 mL of the sample, with continuous stirring for 20 min. Supernatants were filtered through Whatman No. 42 filter paper and diluted 100-fold. Ca in the acid filtrates was determined by a colorimetric method based on the formation of a complex with o-cresolphthalein complexone, in the presence of 8-hydroxyquinoline to prevent interference by Mg (Connerty and Briggs, 1966). The concentration of total P$_i$ in the supernatants was measured in the same filtrates using the colorimetric method of Fiske and Subbarow (1925).

Electron Microscopy. Raw and processed milks were examined using a H-700 Hitachi transmission electron microscope (TEM). Milk samples were diluted 1:40 in 0.02 M CaCl$_2$ and placed in the electron microscope, operating at 100 kV. Micrographs were made at 20000× or 100000× magnification using a 50 μm objective aperture.
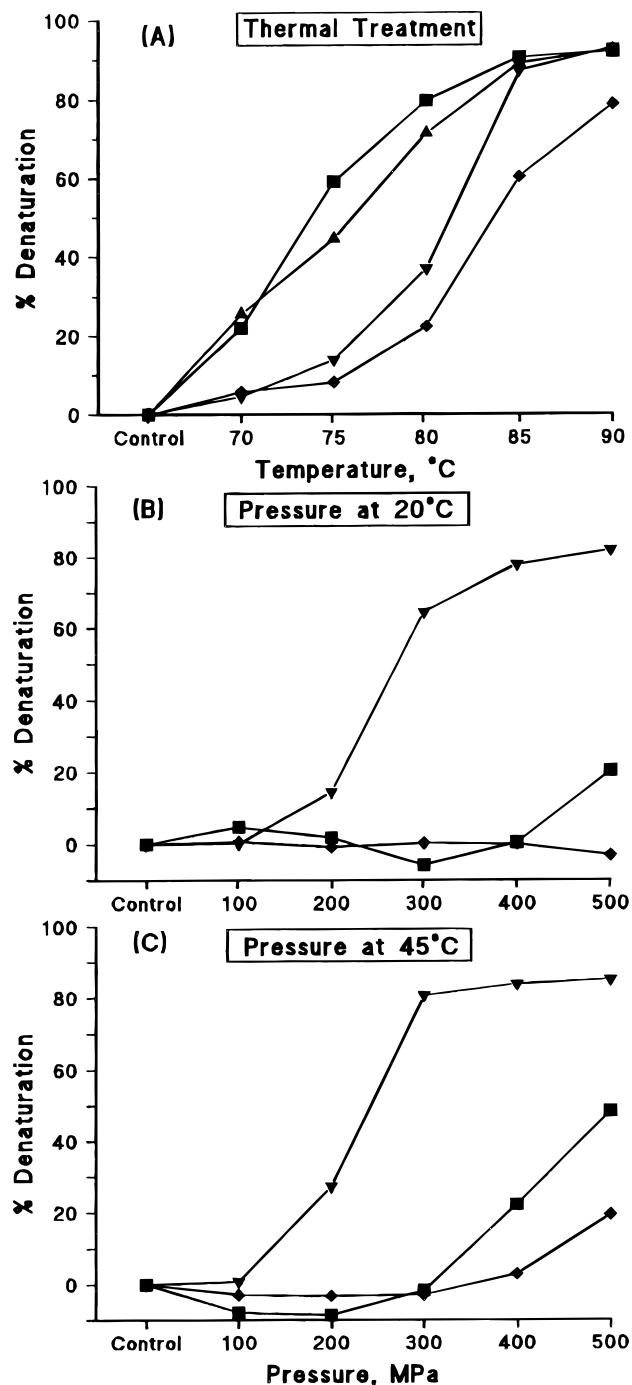

Figure 1. Effect of processing treatments on the extent of denaturation of each whey protein fraction, determined by gel permeation FPLC of acid filtrates from raw and treated milk. (■) Ig, (▲) SA/Lf, (▼) βLg, (♦) αLa. (A) Thermal treatments for 5 min. (B) Pressure treatments at 20 °C for 10 min. (C) Pressure treatments at 45 °C for 10 min.

RESULTS

The effects of thermal and pressure treatments on whey protein denaturation as determined by loss of solubility at pH 4.6 were examined by gel permeation FPLC (Figure 1). As reported previously (Felipe et al., 1997), the sensitivity of the main whey protein fractions (Ig, SA/Lf, βLg, αLa) to denaturation was different for pressure and thermal treatments. Thermal treatments caused the progressive loss of solubility of all the whey proteins, especially the Ig and SA/Lf fractions (Figure 1A). However, pressure treatment at 20 °C (Figure 1B)
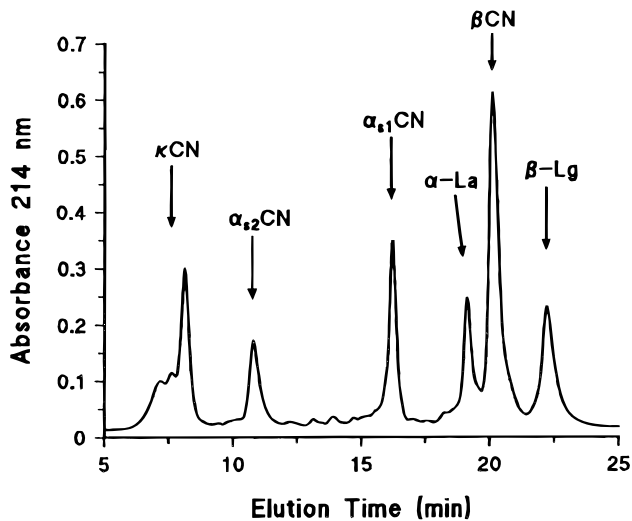
Figure 2. Elution profiles obtained by reverse-phase HPLC on an Apex WP ODS column, showing separation of the caprine caseins and major whey proteins.

resulted mainly in the denaturation of βLg, which initially formed some small, soluble aggregates with the same molecular weight as the SA/Lf. These soluble aggregates caused an apparent increase in the area of the SA/Lf fraction (results not shown). At 400 MPa, most of the βLg had aggregated, but the other major whey protein fractions were largely undenatured. αLa was not affected by pressure treatment at 20 °C, and only partial denaturation of the Ig fraction was observed at 500 MPa. At 45 °C, an increase in the levels of denaturation of βLg was observed above 100 MPa (Figure 1C). The increase in the peak area of the SA/Lf fraction was smaller at this temperature than at 20 °C, indicating that βLg rapidly formed more insoluble aggregates, probably with a higher molecular weight. At 300 MPa, most of the βLg was aggregated, but other fractions were unaffected. At 500 MPa, the Ig and αLa fractions were denatured to differing degrees but to a lesser extent than βLg.

The effect of heating or pressure treatment at 20 and 45 °C on the net negative and positive charges on the caprine caseins was studied by anion- and cation-exchange FPLC, respectively (results not shown). No changes in the profiles were detected after pressure treatment of milk up to 500 MPa, showing that there were no irreversible changes in the net charges or in levels of proteolysis. This result confirms previous reports that pressurization does not break covalent bonds.

Reverse-phase HPLC was used to examine changes in protein concentrations following thermal and pressure treatment, and a typical separation of the proteins in goat's milk is shown in Figure 2. Quantification of the major whey proteins and caseins was possible from a single separation. Structural changes in caprine casein micelles were monitored by determining the amounts of caseins present in the supernatants obtained by centrifuging at different speeds (Figure 3). Centrifugation at 7000g caused only the larger casein micelles to sediment. At 20000g, medium-sized micelles were sedimented, and at 50000g most of the casein micelles were in the pellet. Heating milk for 5 min at temperatures up to 90 °C caused a slight decrease in the amount of serum casein (Figure 3A). This was especially noticeable at 50000g. In contrast, pressure treat-
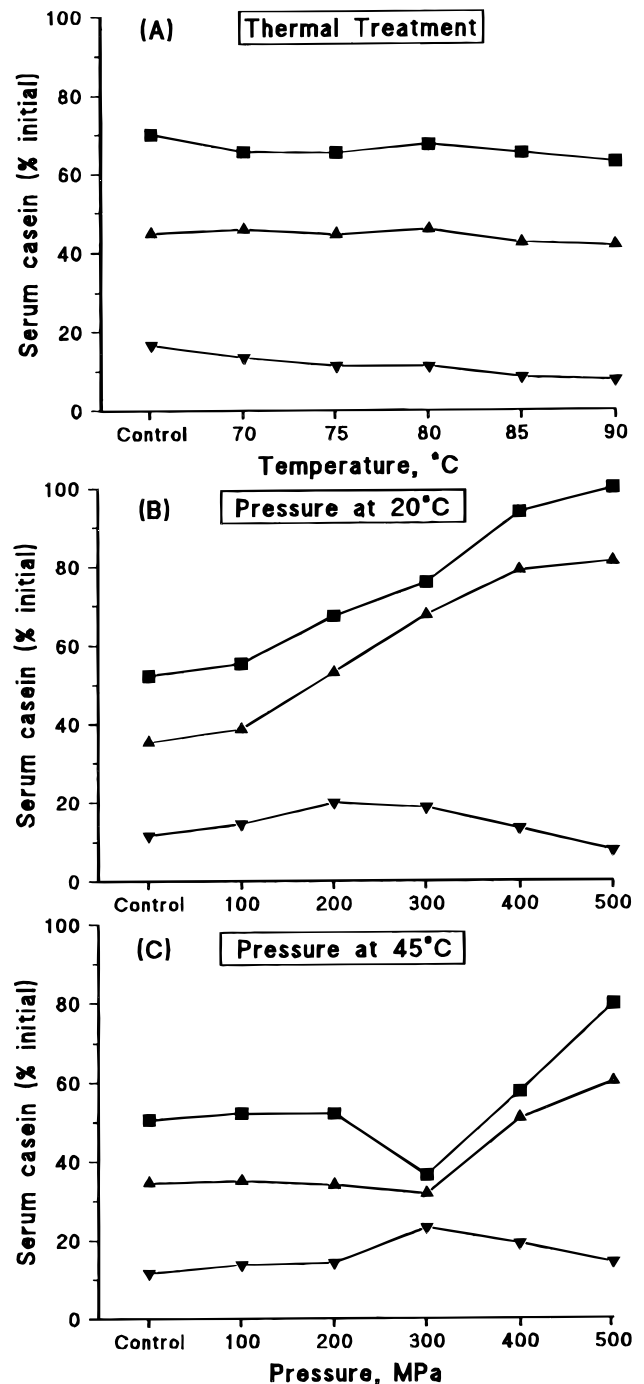

Figure 3. Effect of processing treatments on the amount of serum casein determined by reverse-phase HPLC on the supernatants obtained by ultracentrifugation: (■) 7000g for 30 min, (▲) 20000g for 30 min, (▼) 50000g for 120 min. (A) Thermal treatments for 5 min. (B) Pressure treatments at 20 °C for 10 min. (C) Pressure treatments at 45 °C for 10 min.

ment at 20 °C (Figure 3B) produced a marked increase in the amount of serum casein at 7000 and 20000g. The amount of serum casein increased progressively as pressure increased from 100 to 500 MPa. After treatment at 500 MPa, none of the casein was sedimented by centrifuging at 7000g. This indicated that there was a progressive reduction in the mean micellar size. The amount of serum casein after centrifugation at 50000g increased slightly to a maximum between 200 and 300 MPa, after which it decreased to a level lower than that in the control. Pressure treatment at 45 °C had little

effect on the sedimentation of casein up to 200 MPa (Figure 3C). At 300 MPa, there was a reduction in the level of serum casein at 7000g, which indicated the formation of large micelles that were sedimented very easily. Increasing pressure above 300 MPa, however, increased the amount of serum casein at both 7000g and 20000g, again indicating a reduction in the mean micellar size similar to that at 20 °C.

The concentration of individual caseins and whey proteins in the supernatants obtained after centrifuging the thermal and pressure treated samples at low and high speeds are shown in Figures 4 and 5, respectively. At low centrifugation speeds (Figure 4), sedimentation of α_{S1}-, α_{S2}-, and β-caseins was more pronounced than that of κ-casein, possibly indicating that the sedimentation of large micelles that contained a lower proportion of κ-casein was occurring. Following thermal treatment (Figure 4A) but not after pressure treatments, the whey proteins sedimented at lower centrifugation speeds, most probably due to their association with the surface of sedimented micelles. Pressure treatment of milk at 20 °C caused an increase in the amount of the individual caseins in the supernatant obtained by centrifugation at 7000g (Figure 4B), probably due to a reduction in micellar size. When milk was pressure treated at 45 °C, no changes were observed in protein sedimentation at 7000g with pressures up to 200 MPa (Figure 4C). However, at 300 MPa, the amount of the α_{S1}-, α_{S2}-, and β-caseins in the serum decreased although the amount of κ-casein in the supernatant remained fairly constant, again suggesting that sedimentation of very large micelles had occurred. At higher pressures, the increase in concentrations of the individual caseins in the serum showed a trend similar to that obtained by pressurization at 20 °C although more limited.

Whey protein sedimentation was observed in treated milks after centrifugation at 50000g, especially after thermal treatments (Figure 5A). The decrease in the concentration of the soluble whey proteins was more marked after pressure treatment at 20 °C (Figure 5B) than at 45 °C (Figure 5C), with significant sedimentation occurring only at higher pressure. Heating above 70 °C also reduced the level of serum κ-casein. In contrast, pressure treatment initially increased the concentration of serum κ-casein. This effect was specially marked in samples pressure treated at 45 °C (Figure 5C) where, although most of the other caseins sedimented, κ-casein remained mainly in the serum phase. Pressures higher than 300 MPa caused a decrease in the concentration of serum κ-casein, probably as a result of interaction with whey proteins.

Electron microscopy was used to examine changes in the casein micelles following pressure treatment at 20 and 45 °C, and the results are shown in Figure 6 in comparison with a control skim milk (Figure 6F). Electron microscopy confirmed the results of the centrifugation studies, which showed changes in micellar size on pressure treatment at 20 °C. Increasing pressure up to 500 MPa at 20 °C caused disruption of micelles into smaller particles (Figure 6A,C). This is shown at higher magnification in Figure 6E. At 45 °C, pressure treatment gave an increase in micellar size until no small micelles could be detected, especially between 300 and 400 MPa (Figure 6B). At higher pressures, micellar size again decreased (Figure 6D), and the appearance of the samples was similar to those observed after pressurization at 20 °C.
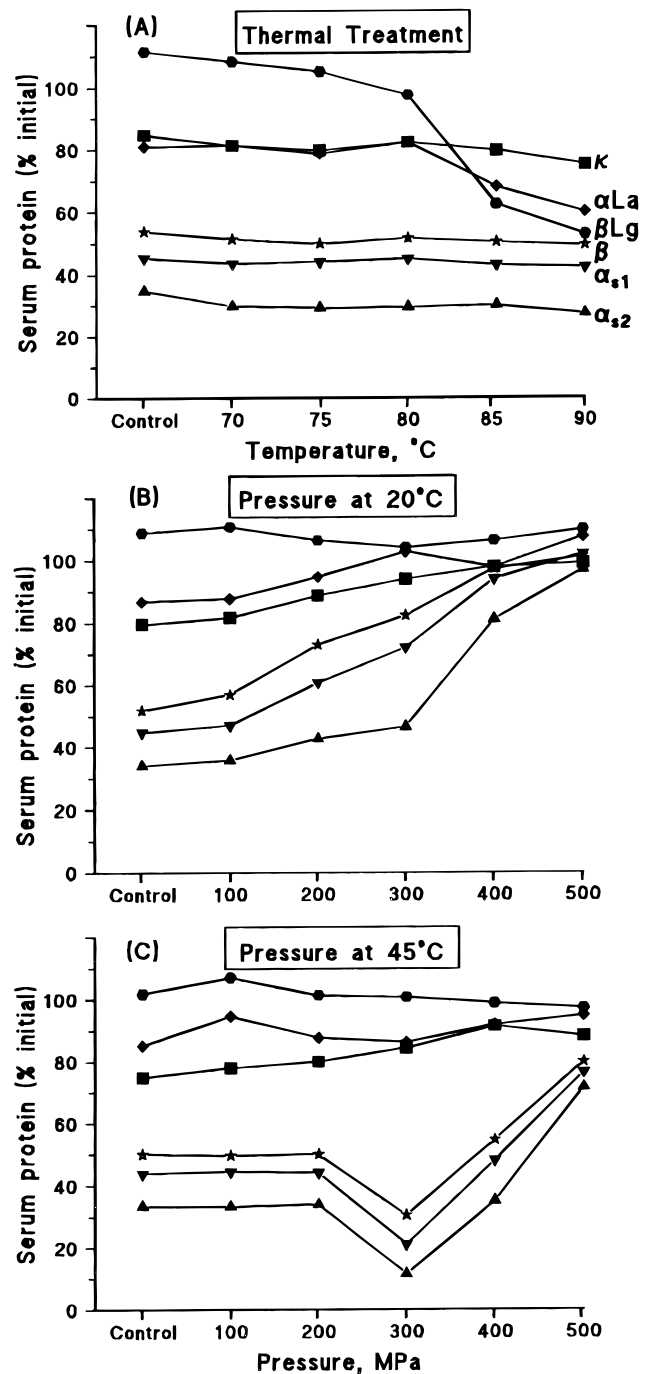

Figure 4. Effect of processing treatments on the amount of serum proteins determined by reverse-phase HPLC on supernatants after centrifugation at 7000g for 30 min (●) βLg, (♦) αLa, (■) κCN, (★) βCN, (▼) α_{S1}, and (▲) α_{S2}. (A) Thermal treatments for 5 min. (B) Pressure treatments at 20 °C for 10 min. (C) Pressure treatments at 45 °C for 10 min.

The effect of thermal and pressure treatments on the distribution of Ca and P_i between the serum and colloidal phases was also studied. Total Ca and P_i concentrations of the supernatants obtained by centrifugation at 50000g were determined. As shown in Table 1, heating milk produced a slight increase in the Ca concentration of the colloidal phase. However, pressure treatment had no significant effect on levels of colloidal Ca. Similar results were observed for P_i concentrations, and the ratio of Ca to P_i in the colloidal phase was maintained at about 2 after each treatment.

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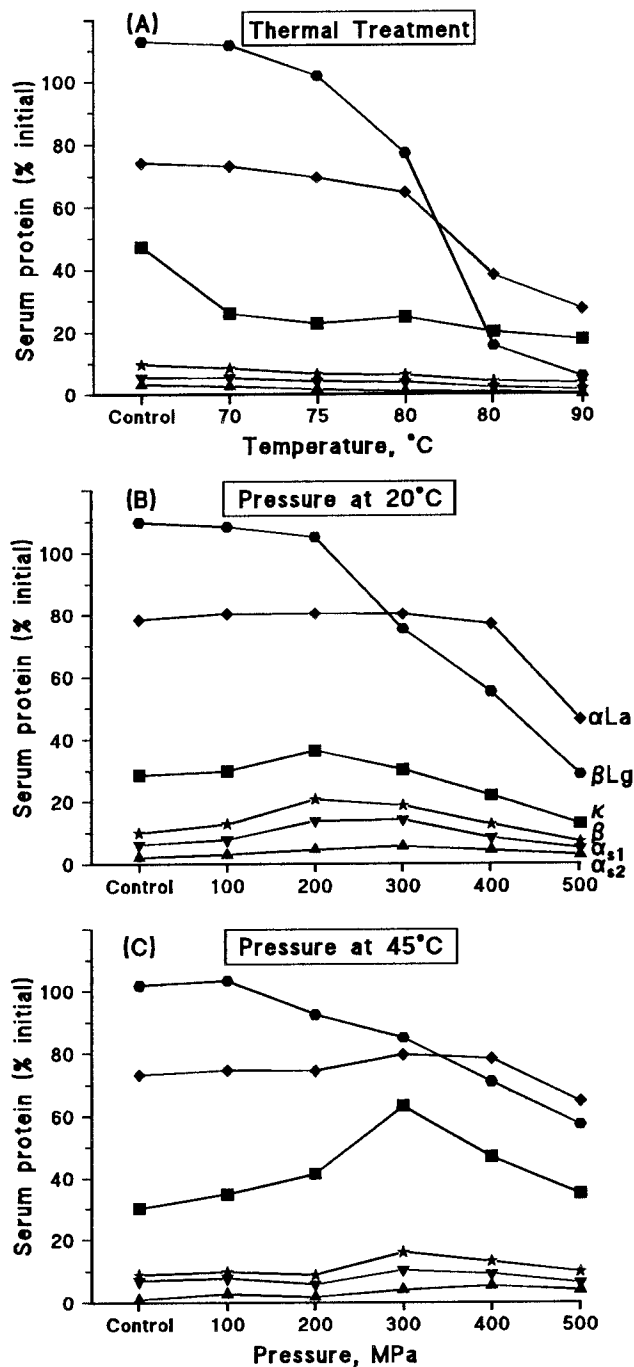*J. Agric. Food Chem.,* Vol. 46, No. 7, 1998 **2527**

Figure 5. Effect of processing treatments on the amount of serum proteins determined by reverse-phase HPLC on supernatants after centrifugation at 50000g for 120 min. (●) βLg, (♦) αLa, (■) κCN, (★) βCN, (▼) α_{S1}, and (▲) α_{S2}. (A) Thermal treatments for 5 min. (B) Pressure treatments at 20 °C for 10 min. (C) Pressure treatments at 45 °C for 10 min.

The composition of protein in the opalescent layer obtained above the pellet by centrifugation at 50000g after thermal treatment or pressure treatment at 20 °C is shown in Figure 7. In control milks, the opalescent layer as compared with the overall composition of the protein in milk was enriched in κ- and β-caseins and poor in α_{S1}- and α_{S2}-caseins. Thermal treatment did not alter the proportions of α_{S1}- and κ-caseins but increased the proportion of β-casein, with a corresponding decrease in the proportions of whey proteins and α_{S2}-casein.

Pressure treatment at 20 °C reduced the proportion of κ-casein and αLa in the opalescent layer and increased the relative amounts of β-, α_{S1}-, and α_{S2}-caseins. The level of βLg was constant. In addition, increasing pressure caused a considerable increase in the volume of the opalescent layer and thus in the total amount of protein in this fraction.

The concentrations of Ca and P$_i$ in the opalescent layer were also determined (results not shown). Thermal treatment did not alter the levels of these two materials in the opalescent layer material. However, pressure treatments caused an increase in Ca and P$_i$, paralleling the increase in total protein and indicating that these two factors are possibly related.

DISCUSSION

The results presented show that, in contrast to thermal treatment where partitioning of casein between the supernatant and pellet phases of centrifuged samples was not affected, pressure treatment at 20 and also at 45 °C at pressures greater than 200 MPa had a significant effect on the sedimentation behavior of the micellar protein. Results from both ultracentrifugation and electron microscopy show that increasing pressure at 20 °C promoted the disruption of the casein micelles and caused the formation of smaller, more slowly sedimenting fragments. At 45 °C, pressure treatment between 300 and 350 MPa produced casein aggregates larger than the micelles in the original milk. Higher pressures at 45 °C, however, caused disruption of the micelles with the formation of smaller, less easily sedimented fragments. Studies on cow's milk have shown that pressure causes disaggregation of micelles resulting in the formation of chains of clusters (Shibauchi et al., 1992), but the mechanism is unclear. Light transmission measurements indicate that there are two steps in the process (Kromkamp et al., 1996). During pressurization, there is an increase in light transmission that is proportional to both the pressure and the duration of treatment, and this shows that there is a decrease in micellar size. When the pressure is released, light transmission decreases rapidly but to a level higher than the initial value. These pronounced changes indicate that the micellar structure is extensively disrupted during pressure treatment.

Current models of micellar structure indicate that the caseins interact with each other through a combination of hydrophobic, electrostatic, and van der Waals forces to form submicelles. The individual submicelles are linked through interactions with calcium and phosphate to form micelles. The present results indicate that pressure treatment influences the various types of forces to differing degrees and, depending on the conditions, can result in disruption or aggregation of micelles. The equilibrium between colloidal and soluble calcium is affected by factors such as temperature, pH, and pressure. Lee et al. (1996) and Anema et al. (1997) showed that the integrity of the colloidal nature of calcium caseinate was dependent on both the applied pressure and the calcium content. Increasing the concentration of calcium in the soluble phase decreased the tendency for the micelles to disaggregate. Gross and Jaenicke (1994) showed that pressure treatment promotes ionization of charged groups, which results in a decrease in pH. This will result in a shift in the calcium equilibrium toward the soluble form. This may in part be responsible for disaggregation of the micelles. In the present
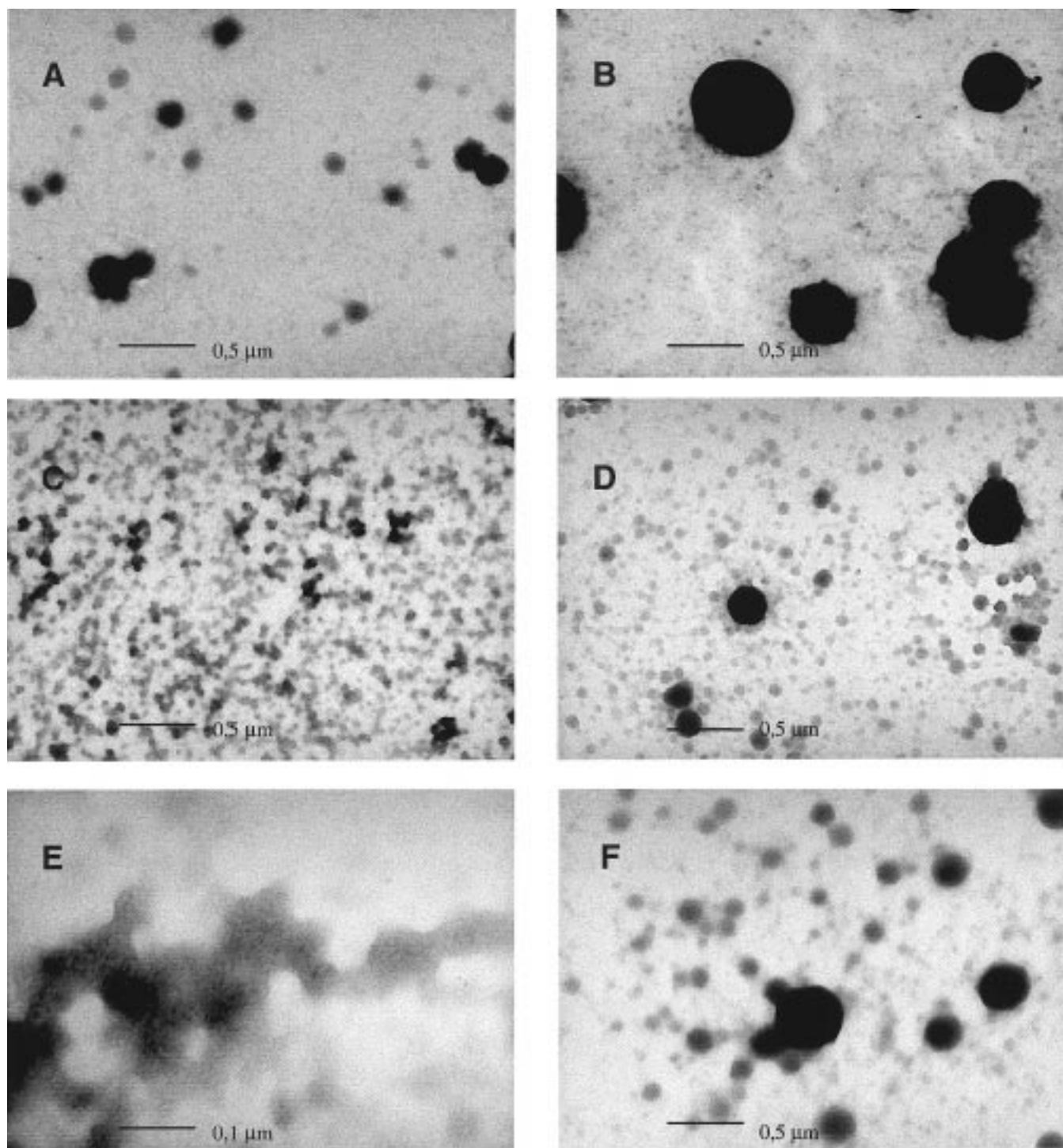
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Figure 6. Effect of pressure on casein micelles in goat's skim milk as observed by transmision electron microscopy. Panels A−D and F were obtained using 20000× magnification. Panel E was obtained using 100000× magnification. Milk pressurized at (A) 300 MPa for 10 min at 20 °C, (B) 350 MPa for 10 min at 45 °C, (C and E) 500 MPa for 10 min at 20 °C, (D) 500 MPa for 10 min at 45 °C, and (F) control skim milk.

study, after pressure treatment, levels of colloidal Ca and P_i were similar to those in the control milk, and this suggests that, when the pressure is released, the calcium equilibrium rapidly shifts back toward the colloidal phase, promoting the formation of new micellar structures. However, since these micelles are formed under different conditions from the original, the structures are unlikely to be the same. For instance, the form of the calcium phosphate may be different and therefore less able to link the submicelles together, and the structure of the submicelles may also be modified.

The temperature at which pressure treatment was performed played an important role in the micellar structural changes. At 45 °C there was a marked difference between the effects of pressure on the behavior of κ-casein and of the other, more hydrophobic α_{S1}-, α_{S2}-, and β-caseins. At 300 MPa, very large micelles that were almost completely devoid of κ-casein were formed. These were unstable and after refrigeration for 24 h, they precipitated. At higher pressures, very large micelles were not formed, and a combination of small micelles and clusters of submicelles similar to those

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Table 1. Effect of Heat and Pressure Treatments on Concentrations of Colloidal Calcium (Ca) and Inorganic Phosphate (P$_i$)

	control	heat (°C)				
		70	75	80	85	90
Ca (mM)	22.24	22.52	23.09	23.20	23.84	24.05
P$_i$ (mM)	10.53	11.05	11.58	11.58	11.58	11.97
ratio Ca/P$_i$ (mM)	2.11	2.03	1.99	2.00	2.06	2.01

	control	pressure at 20 °C (MPa/10 min)				
		100	200	300	400	500
Ca (mM)	23.48	22.79	21.47	22.02	22.72	22.79
P$_i$ (mM)	11.04	11.78	10.00	10.00	12.02	11.76
ratio Ca/P$_i$ (mM)	2.12	1.93	2.14	2.20	1.89	1.94

	control	pressure at 45 °C (MPa/10 min)				
		100	200	300	400	500
Ca (mM)	21.11	20.91	21.05	19.74	20.27	20.59
P$_i$ (mM)	10.26	10.04	10.17	9.77	9.89	10.17
ratio Ca/P$_i$ (mM)	2.05	2.08	2.01	2.02	2.05	2.02
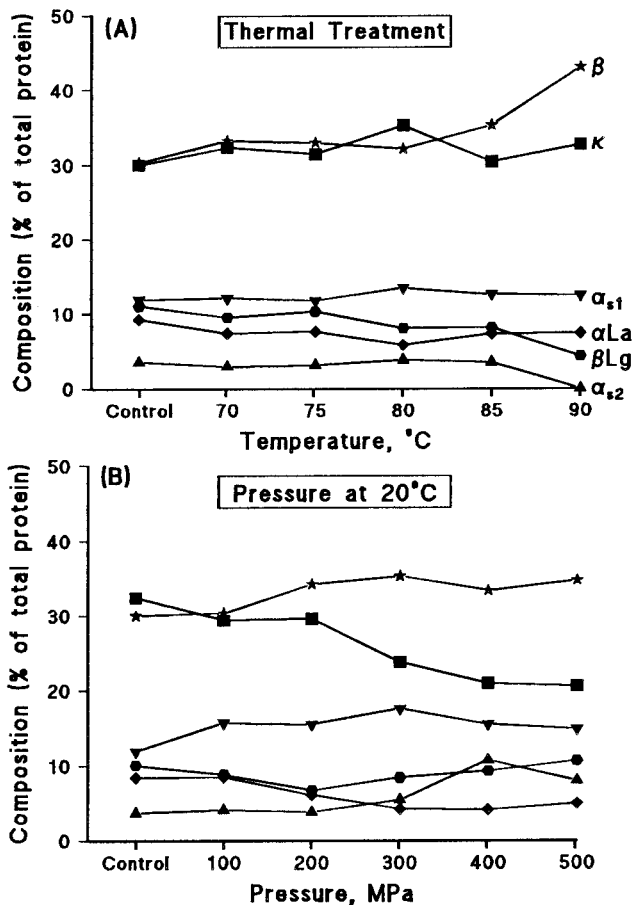
Figure 7. Effect of processing treatments on the proportion of each protein in the opalescent layer of raw and processed milks determined by reverse-phase HPLC. (●) βLg, (♦) αLa, (■) κCN, (★) βCN, (▼) α$_{S1}$, and (▲) α$_{S2}$. Thermal treatments for 5 min. Pressure treatments at 20 °C for 10 min.

formed at 500 MPa and 20 °C were observed. At 45 °C, most of the more hydrophobic caseins were sedimented at 50000*g*, but much of the κ-casein remained in the serum phase, particularly at 300 MPa where it reached a maximum. Above this pressure, the κ-casein content in the serum phase decreased, probably as a result of interaction with whey proteins. At this pressure, κ-casein and whey proteins were not sedimented and separated from the other caseins, and very unstable

micelles were formed that began to precipitate when milk was stored for 24 h.

Both heat and pressure treatments caused denaturation of whey proteins, but there are a number of differences in the processes. Thermal treatment initially caused denaturation, which was followed by interaction with other proteins, the temperature of this effect being specific for each of the whey proteins. The order of ease of thermal denaturation on the basis of loss of solubility at pH 4.6 was Ig > SA/Lf > βLg > αLa. On pressure treatment, βLg was most easily denatured, and appreciable denaturation of Ig and αLa only occurred at higher pressures and more so at 45 °C. Pressure or heat treatment of isolated βLg in buffer sytems results in the formation of small, soluble aggregates (Laligant et al., 1991; Funtenberger et al., 1995). Heating milk, however, causes the formation of insoluble aggregates of βLg, mainly because of interaction with κ-casein on the surface of the micelles, and soluble aggregates are not detected. On pressure treatment, however, βLg initially tends to self-aggregate and only later forms insoluble aggregates with κ-casein (Felipe et al., 1997).

The extent to which the whey proteins affect the formation of the new aggregates of casein on pressure treatment is not known, but the decrease in their solubility at pH 4.6 after pressure treatment suggests that they associate with the micelles. The reported increase in duration of the initial phase of renneting after treatment of cow's milk at pressures above 300 MPa (López-Fandiño et al., 1997), which coincides with the highest level of βLg denaturation, indicates that, as after thermal treatment, βLg associates with micellar κ-casein and tends to inhibit the action of rennet on κ-casein.

Studies on cow's milk have shown that pressure treatment, in addition to affecting the rennet coagulation properties (López-Fandiño et al., 1996), may also change the gel rigidity (Johnston et al., 1993), and further work is required to ascertain the effect of pressure treatment on the renneting and gelling properties of goat's milk.

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