Effect of the high-pressure-release phase on the protein composition of the soluble milk fraction

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ABSTRACT

This paper describes a study on the influence of the high-pressure-release rate on the protein distribution between the soluble and colloidal fractions of milk. Skim milk, without or with sulfhydryl-blocking agents, was pressure treated at 250 and 350 MPa for 5 to 15 min at 25°C, applying different pressure-release rates (pressure-release times between 0.07 to 10 min). The liberation of caseins to the soluble phase and the denaturation of whey proteins were assessed. A significantly higher increase in the content of soluble casein took place during the pressure-release phase as compared with a pressure-holding phase. Denaturation of β -lactoglobulin mainly took place by -SH-S-S exchange reactions during the holding phase. The present results, thus, show a negligible influence of whey proteins on the increase in the content of nonsedimentable casein in pressure-treated milk and provide evidence for the importance of the pressure-release rate in this process, so that the slower the pressure release rate, the higher the level of soluble casein.

Key words: high-pressure-release rate, milk protein, high-pressure treatment, protein distribution

INTRODUCTION

The properties of casein micelles in high-pressure-treated milk related to their size, number, composition, and hydration are different from those of raw or heated milk (Huppertz et al., 2006a; López-Fandiño, 2006). High-pressure treatment brings about the disintegration of casein micelles, which can subsequently reaggregate under certain conditions. The extent of these changes depends on different factors, such as pressure level, holding time, temperature, calcium content, and pH, among others (Arias et al., 2000; García-Risco et al., 2000; Huppertz et al., 2004a). Furthermore, high pressure also denatures whey proteins, which may as-

sociate with casein micelles or micellar fragments, and some studies have dealt with the interaction between both phenomena (Huppertz et al., 2004b; Anema et al., 2005a; Huppertz and de Kruif, 2007).

Although the pressure buildup rate has been reported to have no significant influence on the pressure-induced modification of caseins (Merel-Rausch et al., 2006), the pressure-release rate appears as a factor largely ignored in most high-pressure studies. However, previous results obtained in our laboratory show that the pressure-release rate could be of major importance in terms of micellar disintegration and casein solubilization, as well as in whey protein denaturation (unpublished results). According to Fertsch et al. (2003), the firmness of pressure-induced gels made at 600 MPa from micellar casein solutions depends on the decompression rate, so that the faster the pressure release, the finer the microstructure and the firmer the gels. These authors suggested that once the pressure-holding time brings about the dissociation of the casein micelles, a slow pressure-release rate allows the development of casein hyperstructures, due to the reformation of calcium bridges and hydrophobic interactions, which interfere with homogeneous gelling (Fertsch et al., 2003). Merel-Rausch et al. (2007) also found less-firm gels at slow pressure-release rates. In this case, the increase in the viscosity of 5% micellar casein solutions, submitted to 400 and 600 MPa at 20 and 30°C and fast pressure-release rates (600 MPa/min), was correlated with the formation of loose and large aggregated structures that build from smaller casein micelles. Thus, high pressure-release rates (600 MPa/min) would lead to large hydrodynamic diameters, whereas low pressure-release rates (20 MPa/min) would decrease the hydrodynamic diameter with respect to control milk (Merel-Rausch et al., 2006). In this respect, the influence of whey protein denaturation on pressure-induced casein dissociation or reaggregation is not yet clear (Huppertz et al., 2006a; López-Fandiño, 2006).

In view of the importance that the physicochemical properties of milk proteins have on its technological quality, our aim was to study the influence of the pressure-release rate on the protein distribution between the soluble and colloidal fractions of high-pressure-

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treated milk. For this purpose, skim milk, without or with a sulfhydryl-blocking agent, was pressure treated at 250 and 350 MPa for 5 to 15 min at 25°C, applying different pressure-release times between 0.07 to 10 min. The extent of casein liberation to the soluble phase and the influence of whey protein denaturation on this process were assessed.

MATERIALS AND METHODS

Sample Preparation

Raw bovine milk was collected from a local farm. The milk was skimmed by heating at 37°C for 30 min, centrifugation at $3,000 \times g$ for 30 min at 20°C in a Sorvall Evolution RC centrifuge (Kendro Laboratory Products GmbH, Hanau, Germany), and filtering through glass wool.

Skim milk was treated with N-ethylmaleimide (NEM; Sigma-Aldrich, St. Louis, MO), according to Needs et al. (2000), to block protein sulfhydryl groups involved in whey protein denaturation. N-Ethylmaleimide is insoluble in water, so it was dissolved in dimethyl formamide (DMF; Lab-Scan, Gliwice, Poland) at a concentration of 20 mg/mL and 16.7 mL/L was added to skim milk. Samples with the same volume of DMF added were used as controls. All NEM- and DMF-added samples were incubated overnight at 20°C before the pressure treatments.

High-Pressure Treatments

Samples were poured into 70-mL polyethylene bottles, leaving no headspace, and pressurized at 250 or 350 MPa and 25°C in an ACB 900 HP apparatus (Eurotherm Automation SAS, Lyon, France), with water as a pressure transmitting medium. To assess the effect of the holding time at each pressure level, the pressure was raised at a rate of 2.5 MPa/s; maintained for 5, 6, 10, or 15 min; and released in 0.07 min (4 s); that is, at pressure release rates of 62.5 and 87.5 MPa/s for treatments at 250 and 350 MPa, respectively. To assess the effect of the pressure-release rate, the pressure was increased at a rate of 2.5 MPa/s, maintained for 5 min, and released in 0.07, 1, 5, or 10 min. That is, for treatments at 250 MPa, the pressure release rates were 62.5, 4.2, 0.8, or 0.4 MPa/s and, at 350 MPa, 87.5, 5.8, 1.2, or 0.6 MPa/s, respectively. In the next sections, the pressure-release times will be used as an indicator of the different pressure-release rates to simplify the presentation of the results. All high-pressure treatments were performed at least in duplicate.

Protein Fractions for Analysis

Ultracentrifugation supernatants were obtained from high-pressure-treated skim milk samples by ultracentrifugation at $100,000 \times g$ for 1 h at 20°C in a Beckman L70 preparative ultracentrifuge (Beckman Instruments Inc., San Ramon, CA), using a type 70 Ti rotor, and the supernatants were filtered through Whatman No. 41 filter paper. Fractions soluble at pH 4.6 were obtained by dropwise addition of 2 M HCl under continuous stirring, followed by a 20-min standing period at room temperature, centrifugation at $4,000 \times g$ and 20°C for 30 min, and filtration through Whatman No. 40 filter paper.

Analyses of Milk and Protein Fractions

Determination of Protein Content. The total protein content was determined by the Kjeldahl method, according to the reference procedure published by the International Dairy Federation (FIL-IDF, 1993). In the case of samples with sulfhydryl-blocking agents, the nitrogen content due to the amount of NEM and DMF added was subtracted before its conversion into protein.

Capillary Electrophoresis. Capillary electrophoresis separations were performed using a Beckman P/ACE System 2050 and a TSP-coated fused-silica capillary (BGB Analytik Vertrieb, Schloßböckelheim, Germany) of 57 cm (effective length of 50 cm), 0.50 μm i.d., and slit opening of $100 \times 800 \mu\text{m}$, at 45°C, with a linear gradient from 0 to 25 kV in 3 min, followed by a constant voltage of 25 kV for 47 min. The injection time was 60 s and detection was at 214 nm. Protein identification and quantification was carried out according to Recio et al. (1997).

Statistical Analysis

Results from 2 independent experiments were expressed as mean values $\pm$ standard deviation and were analyzed by a one-way ANOVA to test the influence of the holding time and the pressure-release time and by a 2-way ANOVA to test the interaction between both factors. Statgraphics Plus 5.0 for Windows software was used for data processing (Statistical Graphics Corp., Princeton, NJ; <http://www.statgraphics.com>).

RESULTS

The contents of nonsedimentable protein and pH 4.6-soluble protein of milk high-pressure-treated at 250 and 350 MPa were determined. Table 1 shows the effect of the holding time with 0.07 min of depressurization

Table 1. Protein contents (g/L $\pm$ SD) of the ultracentrifugation supernatants (US) and pH 4.6-soluble fractions (SP) of control skim milk (0 holding and pressure-release times) and milk pressurized at 250 and 350 MPa and 25°C at different holding and pressure-release times

Holding time (min)	Pressure-release time (min)	250 MPa		350 MPa	
		US	SP	US	SP
0	0	12.05 $\pm$ 0.01 ^{b,D}	8.07 $\pm$ 0.02 ^{a,A}	12.05 $\pm$ 0.01 ^{d,E}	8.07 $\pm$ 0.02 ^{a,A}
5	0.07	12.00 $\pm$ 0.08 ^{b,D}	7.79 $\pm$ 0.06 ^{b,B}	14.88 $\pm$ 0.08 ^{c,D}	7.24 $\pm$ 0.04 ^{b,B}
6	0.07	12.83 $\pm$ 0.10 ^{ab}	7.85 $\pm$ 0.38 ^{ab}	15.24 $\pm$ 0.10 ^b	7.22 $\pm$ 0.02 ^b
10	0.07	12.93 $\pm$ 0.88 ^{ab}	8.06 $\pm$ 0.01 ^a	16.16 $\pm$ 0.01 ^{a*}	6.93 $\pm$ 0.11 ^c
15	0.07	13.30 $\pm$ 0.29 ^{**}	8.32 $\pm$ 0.15 ^a	16.24 $\pm$ 0.05 ^{a*}	6.86 $\pm$ 0.10 ^c
5	1	13.06 $\pm$ 0.20 ^C	7.71 $\pm$ 0.08 ^B	15.65 $\pm$ 0.10 ^C	7.36 $\pm$ 0.10 ^B
5	5	14.19 $\pm$ 0.01 ^B	7.65 $\pm$ 0.10 ^B	17.32 $\pm$ 0.31 ^{B*}	7.26 $\pm$ 0.11 ^B
5	10	14.62 $\pm$ 0.11 ^{A*}	7.41 $\pm$ 0.06 ^C	18.44 $\pm$ 0.30 ^{A*}	7.17 $\pm$ 0.06 ^B

^{a-d}Different lowercase superscript letters within a column indicate significant differences ($P < 0.05$) produced by the holding time.

^{A-E}Different uppercase superscript letters within a column indicate significant differences ($P < 0.05$) produced by the pressure-release time.

*Indicates the existence of significant differences ($P < 0.05$) between equivalent values corresponding to the same total treatment times with different combinations of holding and pressure-release times at each pressure level.

and the effect of the depressurization time with 5 min of holding time.

At 250 MPa, the levels of nonsedimentable proteins were similar to that of the control milk at holding times < 15 min, whereas at 350 MPa, they significantly increased with holding times ≥ 5 min (Table 1). The contents of whey proteins soluble at pH 4.6 significantly decreased after 5 min at 250 and 350 MPa, and the holding time at 350 MPa up to 15 min also exerted a significant lowering effect ($P < 0.05$).

As compared with the fastest depressurization (0.07 min), an increase in the pressure-release time (from 1 to 10 min) significantly increased the levels of nonsedimentable proteins at both pressure levels and, particularly, at 350 MPa (Table 1). Total treatment times of 15 min at 250 and 10 and 15 min at 350 MPa led to significantly higher contents of soluble proteins when the holding time was 5 min, with 5 and 10 min of depressurization, than when the holding times were 10 and 15 min, respectively, with 0.07 min of depressurization. However, the pressure-release time and the pressure-holding time exerted a similar influence on the contents of proteins soluble at pH 4.6 after treatments at 250 and 350 MPa (Table 1).

The analysis by capillary electrophoresis of the individual contents of α -LA and β -LG present in the fractions soluble at pH 4.6 of the pressure-treated samples showed that, both at 250 and 350 MPa, the holding time had no effect on the denaturation of α -LA (not shown), but promoted the denaturation of β -LG, particularly at 350 MPa (Figure 1a). In addition, β -LG denaturation was significantly higher ($P < 0.05$) after holding times of 6, 10, or 15 min followed by 0.07 min of release time, than after holding times of 5 min followed by 1, 5, or 10 min of pressure-release time (Figure 1 a and b).

The contents of the individual proteins present in the ultracentrifugation supernatants were analyzed by capillary electrophoresis (Tables 2 and 3). As an example, Figure 2 shows the electropherograms corresponding to samples pressure treated at 350 MPa. At equivalent treatment times, the levels of virtually all individual caseins were significantly ($P < 0.05$) higher when treatments for 5 min at 250 and 350 MPa were followed by 5 and 10 min of depressurization than when milk was subjected to each pressure level for 10 and 15 min, but no differences were observed between the levels of nonsedimentable α -LA or β -LG (Tables 2 and 3).

As shown in Table 4, the hindrance of disulfide-exchange reactions by the addition of NEM did not affect high-pressure-induced protein solubilization, which took place at a higher extent after long depressurization times as compared with long holding times to achieve equivalent treatment times with fast depressurization (0.07 min). On the other hand, as expected, the addition of NEM to milk as a sulfhydryl-blocking agent prevented whey protein denaturation at 350 MPa. The experiments with DMF, in which NEM was dissolved, added to milk, consistently showed the higher effect of the pressure-release time on protein solubilization. However, no significant differences were observed in whey protein denaturation between the holding time and the pressure-release time.

DISCUSSION

According to the present results, considerably large releases of nonsedimentable caseins to the serum phase occurred at 250 and, particularly, at 350 MPa, with the pressure-release time having a greater effect on casein solubilization than the holding time for equivalent treatment times (Tables 1–3; Figure 2). Pressure-

Figure 1. Contents of β -LG soluble at pH 4.6, as measured by capillary electrophoresis, of milk pressurized at 250 (open bars) and 350 MPa (hatched bars). The error bars represent the standard deviation. Lowercase letters (a–c) indicate significant differences ($P < 0.05$) produced by the holding time (a) or the pressure-release time (b) at each pressure level. *Indicates the existence of significant differences ($P < 0.05$) between equivalent values in panels a and b corresponding to the same total treatment time.

induced casein solubilization has been reported to occur at pressures higher than 100 MPa as a result of micellar disruption (López-Fandiño et al., 1998; Hupertz et al., 2004c; Anena, 2008). However, at 250 and 300 MPa, initial disintegration of caseins (during the first 5 to 10 min of treatment) is followed by a progressive aggregation during pressure treatment for prolonged periods due to the enhancement of solvent-mediated or hydrophobic interactions (or both) at these intermediate pressures (Hupertz and de Kruf, 2006; Hupertz et al., 2006b), although it should be noted that treatments at 250 for 5 to 15 min, as those used in

Table 2. Individual proteins present in the ultracentrifugation supernatants control skim milk (0 holding and pressure-release times) and milk pressurized at 250 MPa and 25°C at different holding and pressure-release times¹

Item	Pressure-release time (min)	α -LA	β -LG	α_{S2} -CN	α_{S1} -CN	α_{S1} -CN 9P	κ -CN	β_B -CN	β_{A1} -CN	β_{A2} -CN
Holding time (min)										
0	0	107.93 ^{a,A}	101.34 ^{a,A}	15.05 ^{a,B}	13.30 ^{c,D}	14.96 ^{c,D}	23.19 ^{a,C}	28.88 ^{a,C}	16.98 ^{b,D}	21.39 ^{b,D}
5	0.07	108.47 ^{a,A}	96.24 ^{a,A}	15.78 ^{a,B}	17.09 ^{b,CD}	20.70 ^{ab,C}	26.57 ^{a,BC}	23.16 ^{a,C}	17.15 ^{b,D}	19.20 ^{c,E}
6	0.07	102.96 ^a	93.93 ^a	17.41 ^a	20.00 ^a	22.49 ^a	30.12 ^a	32.72 ^a	21.82 ^a	23.99 ^a
10	0.07	105.97 ^a	93.74 ^a	18.24 ^a	17.74 ^{b*}	19.28 ^{b*}	28.91 ^{a*}	30.54 ^a	19.37 ^{ab*}	23.59 ^{a*}
15	0.07	98.91 ^a	90.72 ^a	21.52 ^a	16.57 ^{b*}	17.74 ^{bc*}	27.51 ^{a*}	25.43 ^a	18.52 ^{b*}	21.14 ^{b*}
5	1	96.59 ^A	91.00 ^A	18.77 ^{AB}	18.38 ^C	20.91 ^C	31.93 ^B	27.45 ^{BC}	20.17 ^C	23.05 ^C
5	5	96.91 ^A	93.05 ^A	19.45 ^{AB}	24.11 ^{B*}	26.62 ^{B*}	37.96 ^{A*}	33.26 ^{AB}	24.65 ^{B*}	27.99 ^{B*}
5	10	109.36 ^A	101.56 ^A	26.01 ^A	29.31 ^{A*}	33.10 ^{A*}	46.62 ^{A*}	35.23 ^A	30.71 ^{A*}	31.23 ^{A*}
Pooled SD ²		3.65	3.28	2.34	0.57	0.86	2.51	3.18	0.71	0.33
Pooled SD ³		3.52	3.46	2.66	1.18	1.22	1.54	1.66	0.60	0.29

^{a-c}Different lowercase superscript letters within a column indicate significant differences ($P < 0.05$) produced by the holding time.
^{A-E}Different uppercase superscript letters within a column indicate significant differences ($P < 0.05$) produced by the pressure-release time.
¹Values are expressed as percentages of the content of each individual casein in control skim milk, estimated by capillary electrophoresis.
²Pooled SD of the values corresponding to control milk and samples pressurized for 5, 6, 10, and 15 min and depressurized in 0.07 min.
³Pooled SD of the values corresponding to control milk and samples pressurized for 5 min and depressurized in 0.07, 1, 5, and 10 min.
*Indicates the existence of significant differences ($P < 0.05$) between equivalent values corresponding to the same total treatment times with different combinations of holding and pressure-release times at each pressure level.

Figure 2. Electropherograms of control skim milk (a) and ultra-centrifugation supernatants of control skim milk (b) and milk pressure treated at 350 MPa and 25°C for 5 min with depressurization in 0.07 min (c). Electropherograms d to f show the effect of the holding time (6, 10, and 15 min) followed by 0.07 min of depressurization and g to i show the effect of the pressure-release time (1, 5, and 10 min) after 5 min of holding time. 1 = α -LA; 2 = β -LG; 3 = α_{s2} -CN; 4 = α_{s1} -CN; 5 = α_{s1} -CN 9P; 6 = κ -CN; 7 = β_B -CN; 8 = β_{A1} -CN; 9 = β_{A2} -CN.

Table 3. Individual proteins present in the ultracentrifugation supernatants control skim milk (0 holding and pressure-release times) and milk pressurized at 350 MPa and 25°C at different holding and pressure-release times¹

Item	Pressure-release time (min)	α -LA	β -LG	α_{s2} -CN	α_{s1} -CN	α_{s1} -CN 9P	κ -CN	β_B -CN	β_{A1} -CN	β_{A2} -CN
Holding time (min)										
0	0	107.93 ^{a,A}	101.34 ^{a,B}	15.05 ^{b,B}	13.30 ^{d,D}	14.96 ^{b,D}	23.19 ^{c,D}	24.88 ^{b,C}	16.98 ^{c,D}	21.39 ^{c,C}
5	0.07	102.61 ^{a,A}	90.57 ^{ab,A}	29.18 ^{a,A}	25.34 ^{c,C}	27.19 ^{a,C}	33.89 ^{b,CD}	35.91 ^{a,B}	25.93 ^{b,C}	29.75 ^{b,B}
6	0.07	100.53 ^a	88.61 ^{bc}	27.66 ^a	26.88 ^{bc}	29.57 ^a	37.94 ^{ab}	37.76 ^a	26.82 ^b	29.58 ^b
10	0.07	95.85 ^a	78.33 ^c	29.75 ^a	29.64 ^{ab}	26.86 ^a	40.51 ^a	34.66 ^{a*}	29.66 ^{a*}	32.56 ^{ab*}
15	0.07	91.26 ^a	79.73 ^{bc}	32.17 ^a	30.29 ^{ab}	30.72 ^{ab}	40.97 ^{a*}	37.45 ^{a*}	31.19 ^{a*}	36.07 ^{a*}
5	1	97.93 ^A	88.62 ^A	30.73 ^A	28.52 ^{BC}	29.56 ^{BC}	38.64 ^{BC}	37.48 ^B	30.01 ^C	35.07 ^B
5	5	100.77 ^A	88.41 ^A	31.24 ^A	32.51 ^B	33.92 ^{AB}	49.35 ^{AB}	45.97 ^{A*}	37.70 ^{B*}	43.06 ^{A*}
5	10	99.89 ^A	88.00 ^A	35.33 ^A	37.91 ^{A*}	39.31 ^{A*}	53.49 ^{A*}	49.00 ^{A*}	44.18 ^{A*}	48.18 ^{A*}
Pooled SD ²		4.65	3.13	2.17	0.87	2.17	1.76	2.38	0.46	1.42
Pooled SD ³		3.79	1.82	2.30	1.23	1.31	3.14	2.22	1.20	1.90

^{a-c}Different lowercase superscript letters within a column indicate significant differences ($P < 0.05$) produced by the holding time.

^{A-D}Different uppercase superscript letters within a column indicate significant differences ($P < 0.05$) produced by the pressure-release time.

¹Values are expressed as percentages of the content of each individual casein in control skim milk, estimated by capillary electrophoresis.

²Pooled SD of the values corresponding to control milk and samples pressurized for 5, 6, 10, and 15 min and depressurized in 0.07 min.

³Pooled SD of the values corresponding to control milk and samples pressurized for 5 min and depressurized in 0.07, 1, 5, and 10 min.

*Indicates the existence of significant differences ($P < 0.05$) between equivalent values corresponding to the same total treatment times with different combinations of holding and pressure-release times at each pressure level.

Table 4. Protein contents (% of total milk protein $\pm$ SD) of the ultracentrifugation supernatants (US) and pH 4.6-soluble fractions (SP) of control skim milk (0 holding and pressure-release times) and milk pressurized at 350 MPa and 25°C at different holding and pressure-release times¹

Holding time (min)	Pressure-release time (min)	US		SP	
		NEM	DMF	NEM	DMF
0	0	32.41 $\pm$ 2.47 ^{b,C}	32.41 $\pm$ 0.96 ^{c,C}	21.08 $\pm$ 4.1 ^{a,A}	24.85 $\pm$ 0.67 ^{a,A}
5	0.07	40.07 $\pm$ 0.35 ^{a,B}	38.24 $\pm$ 0.84 ^{b,B}	19.31 $\pm$ 0.94 ^{a,A}	22.61 $\pm$ 0.94 ^{b,B}
10	0.07	38.74 $\pm$ 3.33 ^{a*}	39.57 $\pm$ 2.60 ^{b*}	20.86 $\pm$ 3.50 ^a	21.00 $\pm$ 0.71 ^b
15	0.07	39.03 $\pm$ 0.18 ^{a*}	44.76 $\pm$ 0.18 ^{a*}	20.64 $\pm$ 0.71 ^a	21.00 $\pm$ 0.47 ^b
5	5	50.78 $\pm$ 1.80 ^{A*}	51.62 $\pm$ 0.82 ^{A*}	20.64 $\pm$ 0.39 ^A	22.30 $\pm$ 0.90 ^B
5	10	52.10 $\pm$ 0.70 ^{A*}	53.95 $\pm$ 1.26 ^{A*}	20.58 $\pm$ 1.21 ^A	23.00 $\pm$ 0.63 ^{AB}

^{a-c}Different lowercase superscript letters within a column indicate significant differences ($P < 0.05$) produced by the holding time.

^{A-C}Different uppercase superscript letters within a column indicate significant differences ($P < 0.05$) produced by the pressure-release time.

*Indicates the existence of significant differences ($P < 0.05$) between equivalent values corresponding to the same total treatment times with different combinations of holding and pressure-release times at each pressure.

¹NEM = N-ethylmaleimide; DMF = dimethyl formamide.

the present study, might be too short to induce micellar reformation (Huppertz and de Kruif, 2007). Moreover, the aggregation of the casein-depleted micellar particles that maintain a certain degree of integrity and some intact colloidal calcium phosphate, mainly due to the reestablishment of hydrophobic bonds, once pressure has been liberated, has been reported (Huppertz et al., 2006b; Orlén et al., 2006; Anema, 2008).

Although pressure-disrupted electrostatic and hydrophobic bonds may start to reform while high pressure is released, our results clearly showed that a significantly higher increase in the content of nonsoluble casein took place during the pressure-release phase as compared with the pressure-holding phase. According to Merel-Rausch et al. (2006, 2007), the pressure-release rate, at pressures higher than 200 MPa, is important for casein modification because slow release rates would favor casein reassociation, whereas fast pressure-release rates would disturb self-association mechanisms, building up completely new structures or aggregates. In principle, substantial levels of dissociated casein could be compatible with a situation that promotes micellar reaggregation. In fact, high levels of nonsedimentable caseins are found in the case the aggregated samples formed at pressures of 250 to 300 MPa (Anema et al., 2005a), an observation that suggests that the aggregation of casein-depleted micelles takes place as a separate event from what causes micellar disruption, even if a minimum level of micellar disruption seems to be required for aggregation to occur (Huppertz and de Kruif, 2006; Anema, 2008).

The present results support previous findings that indicate that β -LG denatures at pressures higher than 100 MPa to an extent that depends on the pressure level and the duration of the treatment (López-Fandiño et al., 1996; Anema et al., 2005b). Reversible unfolding of β -LG, exposure of the hydrophobic regions and

of the free -SH group under pressure can be followed by irreversible denaturation, whether self aggregation or association with other whey proteins or with κ -CN (López-Fandiño et al., 1997; Needs et al., 2000; Patel et al., 2006). The observation that, in milk pressurized at 250 and 350 MPa, a proportion of denatured β -LG remained soluble at the normal milk pH (Figure 1; Tables 2 and 3) has also been made by other authors (López-Fandiño et al., 1998; Anema, 2008), and suggests that β -LG molecules preferentially self-associate or interact with nonsedimentable casein particles. Our results (Table 1; Figure 1) indicate that β -LG denatured mainly due to disulfide linkages during the pressure-holding phase, under conditions of high solubility of calcium phosphate and inhibition of hydrophobic interactions, because little additional denaturation took place during the decompression phase. This supports that the -SH-S-S exchange reactions mainly take place during the pressure treatment (Belloque et al., 2000), suggesting that the hydrophobic bonds that may reform during the pressure-release phase do not play a major role in β -LG denaturation.

The present results also suggest a negligible influence of the whey proteins on the increase in the content of nonsedimentable casein that occurred during the depressurization phase, as this was not accompanied by further changes in the levels of whey proteins present in the ultracentrifugal supernatants (Tables 2 and 3) nor by an increased whey protein denaturation, as determined by the loss of solubility at pH 4.6 (Table 1; Figure 1). As expected, the addition of NEM prevented disulfide bond formation, avoiding most whey protein denaturation and association with caseins (Huppertz et al., 2004d), but it did not have any effect on the release of protein to the soluble phase of milk that took place during the decompression phase (Table 4). Similarly, pressure-induced changes in micellar size are probably

independent of the whey proteins, because similar micellar sizes are reported in high-pressure-treated milk with or without NEM added (Huppertz et al., 2004b). Furthermore, micellar disaggregation and reaggregation have been found to occur at 250 and 300 MPa to comparable extents in the absence or presence of whey proteins (Anema et al., 2005a; Huppertz and de Kruif, 2007).

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