Isolation and Characterization of Copolymers of β -Lactoglobulin, α -Lactalbumin, κ -Casein, and α_{s1} -Casein Generated by Pressurization and Thermal Treatment of Raw Milk

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ABSTRACT

Raw skim milk was submitted to high pressure (300 to 600 MPa) and temperature (4 to 70°C) treatments for 2 or 5 min. The combined effects of pressure and temperature on milk proteins induced structural changes and polymer and copolymer formation characterized by anion-exchange and size-exclusion fast protein liquid chromatography and electrophoretic techniques. Approximately half of the β -lactoglobulin formed polymers, and the other half formed large copolymers, mainly with κ -casein, α -lactalbumin via intermolecular disulfide bond exchange, and α_{s1} -casein via physicochemical interactions, in proportions of 1.0:0.7:0.3:0.1, respectively. Minor whey proteins (serum albumin, immunoglobulins, and lactoferrin) also participated in the formation of the copolymers but to a lesser extent. Two populations of the copolymers were found with apparent molecular masses ranging from 440 to 2000 kDa for the first and more than 2000 kDa for the second. On the contrary, for heated milks the aggregation kinetics obtained by combination of high pressure and thermal treatment were very fast, as no intermediates such as dimers and small size oligomers were observed after pressurization, whatever the temperature studied. Lactosylation of proteins as well as proteolysis were very limited. A β -casein amino-terminal peptide of 22 kDa was specifically recovered in milk samples treated under the more drastic conditions (500 MPa/55°C per 5 min and 600 MPa/70°C per 5 min) and might have been generated by neutral proteases such as elastase released from somatic cells present in milk. No casein was released from the micelle whatever the combination of high pressure and temperature studied.

(**Key words:** high pressure, raw milk, protein aggregation, β -lactoglobulin)

Abbreviation key: C = ratio bisacrylamide on acrylamide plus bisacrylamide, **FPLC** = fast protein liquid chromatography, **T** = concentration of acrylamide plus bisacrylamide.

INTRODUCTION

Pressure-induced protein denaturation is a complex phenomenon, mainly resulting from the disruption of hydrophobic, ionic, and hydrogen interactions (Famelart et al., 1997; Iametti et al., 1997). The extent of pressure-induced changes in proteins depends on many factors such as temperature, pH, solvent composition, and ionic strength, as well as on the native protein structure and applied pressure (Tedford and Schaschke, 2000).

The mechanism of copolymerization of α -LA and β -LG mixtures is governed by the formation of polymers of β -LG, as α -LA does not form polymers when heated alone (Schokker et al., 2000). When mixtures of β -LG and α -LA or BSA are heated to 75°C but not pressurized, polymers of each protein as well as copolymers of these proteins are observed (Havea et al., 2001). Heat-induced aggregation of β -LG occurs via many intermediates (Bauer et al., 1998; Schokker et al., 1999). In early stages of the aggregation of β -LG, nonnative dimers and small oligomers are formed. The first change is the dissociation of the native β -LG dimer into monomers (Schokker et al., 1999). The second change is the partial unfolding of the β -LG monomer, as the alkyl thiol and hydrophobic residues become solvent accessible, resulting in a reactive monomer at high temperature (78.5°C; Schokker et al., 1999). The increase in aggregate size occurs via the addition of reactive monomers, dimers, and small aggregates to larger aggregates. According to Roefs and De Kruif (1994), the model of β -LG aggregation recognizes an initiation, a propaga-

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tion, and a termination step by analogy with polymer radical chemistry. According to Schokker et al. (1999), aggregates with more than one alkyl thiol can be formed.

Following high-pressure treatment of milk at 25°C, there is a decrease in the amount of whey proteins remaining in the solution at pH 4.6, indicating an increase in protein polymerization. After pressure treatment, β -LG is the most easily denatured (for pressure starting from 100 MPa), whereas other isolated whey proteins such as α -LA and BSA are more resistant to pressure (at least 400 MPa during 60 min), probably due to the absence of alkyl thiol (Lopez-Fandino et al., 1996). The denaturation of β -LG studied in solution involves the dissociation of dimers to monomers (having reactive alkyl thiol) together with changes in the conformation of the polypeptide chain (Tedford and Schaschke, 2000). The oligomerization of isolated β -LG and formation of polymers of high molecular mass observed at pH close to 7.0 largely depends on intermolecular disulfide bond exchange (Funtenberger et al., 1995, 1997). The extent of pressure-induced changes on proteins may vary quantitatively or qualitatively depending on media used (i.e., isolated proteins in buffered solutions or whole skimmed milk). Therefore, it is important not to extrapolate results from model systems to complex systems such as raw milk.

In this study, raw skimmed milk was used as the suspending medium. The objective of this work was to investigate the effects of high hydrostatic pressures applied at different temperatures on the structural changes of milk proteins. In particular, copolymers formed under high pressure were isolated by anion-exchange chromatography and characterized by electrophoresis and size-exclusion chromatography.

MATERIALS AND METHODS

Pressurization and Thermal Treatment of Milk

Fresh raw bovine milk containing a natural microflora of approximately 10,000 cfu/mL and a SCC of 309,000/mL was obtained from a local dairy herd of 90 Prim'Holstein cows at the beginning of lactation (days in milk < 120). The cows were in their second through fifth lactation. Milk from 2 milkings was mixed, stored at 4°C, and treated within 12 h of collection. Discontinuous high hydrostatic pressure equipment (ACB Pressure Syst., Nantes, France), including a cylindrical 50-L stainless steel chamber, was used. The cylindrical chamber was fitted with double envelopes within which water circulation ensured temperature regulation. Four 250-mL milk samples were preequilibrated for 10 min at 4, 20, 55, or 70°C and then pressurized at given temperatures (P300 at 300 MPa/4°C per 2 min, P400

at 400 MPa/20°C per 2 min, P500 at 500 MPa/55°C per 5 min, and P600 at 600 MPa/70°C per 5 min). The pressurized milk samples were compared to raw skimmed milk (control).

Fractionation of Milk after Physical Treatment

After pressurization and thermal treatment, milk was fractionated by 2 methods. In the first, lactosoluble proteins were separated from isoelectric CN by acidification at pH 4.6 with 1 M acetic acid and centrifugation (4000 $\times$ g, 20 min, 4°C). In the second, milk was ultracentrifuged (15,000 $\times$ g, 60 min, 4°C) to separate soluble proteins of the supernatant (named lactoplasma) from the micellar CN. The lactosoluble and lactoplasma were dialyzed against distilled water, freeze-dried, and stored at -20°C.

Fast Protein Liquid Chromatography

Proteins were fractionated at room temperature by anion-exchange fast protein liquid chromatography (FPLC; ÅKTA-FPLC model, Amersham, Uppsala, Sweden) on an analytical Mono Q HR 5/5 column (Amersham). Volumes of 200 μ L of lactosoluble or lactoplasma proteins of C, P300, P400, P500, and P600 (5 mg/mL) were loaded onto the column equilibrated in 20 mM Tris/HCl buffer, pH 7.2, containing 0.02% (wt/vol) sodium azide. The flow rate was 1 mL/min, and detection was monitored at 280 nm. A linear gradient from 0 to 0.4 M NaCl was applied from 2 to 22 min. The peak areas were calculated with UNICORN software (Amersham).

The major material present in lactoplasma of P500 with a retention time of 18.8 min was collected, dialyzed, and freeze-dried before size-exclusion FPLC analysis. A volume of 100 μ L of this major material (1 mg/mL) was injected onto the Superose 6 HR 10/30 column (Amersham) connected to the FPLC system. The eluent was 50 mM Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl and 0.02% (wt/vol) sodium azide. The flow rate was 0.25 mL/min, and detection was monitored at 280 nm. The molecular mass standards were dextran blue (2000 kDa), apoferritin (440 kDa), IgG (160 kDa), and BSA (67 kDa).

Electrophoresis

Sodium dodecyl sulfate-PAGE was performed with a 4.9%T 2.7%C polyacrylamide stacking gel (T = concentration of acrylamide plus bisacrylamide; C = ratio bisacrylamide on acrylamide plus bisacrylamide) in 0.125 M Tris/HCl buffer, pH 6.8, containing 0.1% (wt/vol) SDS, and with a 15.4%T 2.7%C polyacrylamide resolv-

ing gel in 0.38 M Tris/HCl buffer, pH 8.8, containing 0.1% (wt/vol) SDS (Laemmli and Favre, 1973). Samples were dissolved at 2 mg/mL in 0.125 M Tris/HCl buffer, pH 6.8, in the presence of 0.1% (wt/vol) SDS, 10% (vol/vol) glycerol, and 0.01% (wt/vol) bromophenol blue, without or with 5% (vol/vol) 2-mercaptoethanol (nonreducing or reducing conditions, respectively). After heating at 100°C for 3 min, 20 μ L of the sample was loaded in the gel (10 μ L for the material eluted by anion-exchange FPLC at 18.8 min). The molecular mass standards (Bio-Rad, Hercules, CA) were myosin (200.0 kDa), β -galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (apparent molecular mass of 15.5 kDa), and aprotinin (6.5 kDa). In all cases, the standards were separated by SDS-PAGE under reducing conditions. Proteins were fixed with 12% (wt/vol) TCA for 30 min and then stained for 60 min by 0.5% (wt/vol) R-250 Coomassie blue dissolved in a mixture of 50% (vol/vol) ethanol and 12% (wt/vol) TCA, followed by an overnight destaining in a solution of 30% (vol/vol) ethanol, 7.5% (vol/vol) acetic acid, and 5% (wt/vol) TCA. Staining of glycoproteins was carried out with Schiff's reagent after 1% (wt/vol) periodic acid oxidation (Kapitany and Zebrowski, 1973) after electrotransfer of the electrophoretically separated bands onto a polyvinylidene difluoride membrane, as previously reported (Egito et al., 2001). Relative proportions of proteins were determined with the GS-800 densitometer equipped with Quantity One software (Bio-Rad).

Alkaline PAGE was carried out with a 9.25%T 3.84%C polyacrylamide gel in 0.38 M Tris/HCl buffer, pH 8.9 (Pâquet et al., 1988). Samples (2 mg/mL) were solubilized in 0.38 M Tris/HCl, pH 8.9, containing 10% (vol/vol) glycerol and 0.01% (wt/vol) bromophenol blue. Volumes of 20 μ L were loaded in the gel. After electrophoresis, proteins were stained by Coomassie blue as described above.

Microsequencing

Amino-terminal microsequencing of bands electrophoretically separated and electrotransferred (as described by Egito et al., 2001) was performed on a Model 476A microsequencer (Applied Biosystems, Foster City, CA) with online identification of the phenylthiohydantoin derivatives.

RESULTS

Fast Protein Liquid Chromatography

The major whey components of the lactosera and lactoplasmas were separated by anion-exchange chro-

matography (Figure 1). Independently of whey protein preparation (lactosera or lactoplasma), P300 (300 MPa/4°C per 2 min) and P400 (400 MPa/20°C per 2 min) displayed chromatographic profiles similar to those of the control (lactosera and lactoplasma proteins prepared from raw milk) with ratios α -LA-to- β -LG in the range of 0.6 to 0.9. The β -LG B content was slightly overestimated in lactoplasma due to the presence of cold-soluble CN, leading to an unresolved peak (lactoplasma profiles; Figure 1). Indeed, the preparation of lactoplasma by ultracentrifugation at 4°C allows more CN, particularly β -CN, to appear as whey proteins (Davies and Law, 1983). β -Lactoglobulin, however, disappeared dramatically on the chromatographic profiles of P500 (500 MPa/55°C per 5 min) and P600 (600 MPa/70°C per 5 min), probably due to more drastic pressurization and thermal treatments. The decrease of the β -LG content was more important in lactosera than in lactoplasma. In lactosera, the α -LA-to- β -LG ratio was 3.0 for P500 and 6.4 for P600, whereas in lactoplasma, the ratio was 1.7 for P500 and 1.8 for P600. The preparation mode of the whey proteins thus had an influence on the solubility of β -LG.

The peak with the highest retention time (18.8 min; Figure 1) was observed in the lactoplasma profiles of all pressurized milk samples P300, P400, P500, and P600, whereas this peak was absent on the lactosera profiles of the corresponding samples and of the lactosera and lactoplasma profiles of the control. As it was observed for β -LG, the components eluted at 18.8 min seemed to coprecipitate with the insoluble material at pH 4.6 but remained in suspension in the supernatant after ultracentrifugation. Their amount in milk increased as the treatment became more drastic (relative proportions of 11.4, 7.3, 67.3, and 74.1% for P300, P400, P500, and P600, respectively) and therefore would correspond to protein complexes. In order to determine their molecular size, the compounds at 18.8 min were collected and submitted to size-exclusion FPLC analysis. These compounds were eluted in the void volume of the Superose 12 column that corresponded to an apparent molecular mass higher than 300 kDa (data not shown). The FPLC analysis on a Superose 6 column showed 2 peaks at and near the void volume that corresponded to 2 populations of aggregates with apparent molecular masses ranging from 440 to 2000 kDa for the first and more than 2000 kDa for the second (Figure 2).

Electrophoresis

The different samples prepared from pressurized milk samples were submitted to SDS-PAGE performed under nonreducing and reducing conditions (Figure 3).

Figure 1. Anion-exchange fast protein liquid chromatography on a Mono Q column at pH 7.2 of lactoserum and lactoplasma proteins of raw milk (control) and pressurized milk samples P300, P400, P500, and P600 (the conditions of pressurization and heat treatment indicated in the figure). Volumes of 200 μ L (lactoserum and lactoplasma proteins at 5 mg/mL) were loaded onto the column. Numbers 1 to 8 refer to fractions collected for further electrophoresis analyses. A and B correspond to the A and B variants of β -LG, respectively.

As expected, the main difference between lactoserum and lactoplasma lay in the absence of CN in lactoserum and in the presence of CN in lactoplasma. The so-called “SDS-monomeric” β -LG (Havea et al., 1998) disappeared almost completely on the nonreducing SDS-PAGE patterns of P500 (lactoserum and lactoplasma patterns) and in totality on those of P600 (Figure 3a), whereas it was recovered in the lactoserum and lactoplasma of P300 and P400. Kappa-casein was recovered on the reducing SDS-PAGE patterns of the P500 and P600 lactoplasma proteins in higher quantity than on the corresponding lactoserum patterns. It is well-known that this casein is a natural polymer from 2 to 8 units associated by disulfide bonds (Farrell et al.,

1998). The κ -CN was, however, in smaller quantity on the reducing SDS-PAGE patterns of the control, P300, and P400 lactoplasma proteins (Figure 3b), suggesting that the presence of κ -CN in lactoplasma needed another explanation.

In the case of the P500 and P600 lactoplasma proteins, the wells at the top of the stacking gel performed under nonreducing conditions were stained by Coomassie blue (Figure 3a) and might correspond to the high-molecular-mass complexes highlighted by size-exclusion FPLC. This staining disappeared after the addition of 2-mercaptoethanol, and relatively high amounts of reduced κ -CN monomers were generated together with reduced β -LG and α -LA monomers (Figure 3b). The

Figure 2. Size-exclusion fast protein liquid chromatography on a Superose 6 column of copolymers isolated from lactoplasma proteins of the pressurized milk P500 (500 MPa/55°C) by anion-exchange fast protein liquid chromatography. Volume of 100 μ L (copolymers at 1 mg/mL) were injected onto the columns. Vo = dead volume determined with dextran blue; MM = molecular mass.

nonreducing and reducing SDS-PAGE patterns of the complexes showed that β -LG, κ -CN, and α -LA formed disulfide crosslinked copolymers associated with a low quantity of α _{s1}-CN by physicochemical interactions, in proportions of 1.0:0.7:0.3:0.1, respectively (Figure 4). The α _{s1}-CN was identified with certainty by amino-terminal microsequencing after electrotransfer (SDS-PAGE alone does not distinguish α _{s1}- and α _{s2}-CN; data not shown). Low amounts of minor proteins (lactoferrin, BSA, and IgG) were also bound to the copolymers by disulfide bond exchange. Indeed, these proteins were absent on the nonreducing SDS-PAGE pattern but present on the reducing SDS-PAGE pattern of the copolymers, whereas the α _{s1}-CN was present on the 2 patterns. Sodium dodecyl sulfate causes disruption of physicochemical interactions between proteins, whereas chemical bonds are unaffected. On the other hand, the presence of α _{s2}-CN in the copolymers was not detected by urea-PAGE performed in the presence of 2-mercaptoethanol (separation of α _{s1}- and α _{s2}-CN is achieved by urea-PAGE; data not shown).

The α -LA-to- β -LG ratios determined for the SDS-monomeric proteins of control, P300, and P400 ranged from 0.5 to 0.7 (Table 1). Under reducing conditions, the ratios for P500 and P600 were approximately twice the ratios of control, P300, and P400 in the lactosera, whereas they were similar in the lactoplasma. Moreover, these α -LA-to- β -LG ratios were twice those of P500 and P600 lactoplasma proteins (Table 1) so that approximately 50% of β -LG formed disulfide cross-linked polymers, and approximately 50% formed copolymers (if almost all the β -LG monomers were polymerized as suggested in Figure 3a and by considering as

Figure 3. Nonreducing (A) and reducing SDS-PAGE (B) of lactosera and lactoplasma proteins of raw milk (control C) and the pressurized milk samples P300 (300 MPa/4°C), P400 (400 MPa/20°C), P500 (500 MPa/55°C), and P600 (600 MPa/70°C). Volumes of 20 μ L (lactosera and lactoplasma proteins at 2 mg/mL) were loaded in the gels. Bands a and b corresponded to cysteinyl-S-propionamide derivative of β -LG and amino-terminal fragment of β -CN, respectively. M = mixture of molecular mass standards reduced by 2-mercaptoethanol. Lf = lactoferrin; PP3 = proteose peptone component-3. Identification of each individual CN (i.e., α _{s1}-CN, β -CN, γ 1-CN, γ 2-CN, and κ -CN) is reported on the SDS-PAGE patterns.

negligible the proportion of β -LG in the CN pellets; data not shown).

In order to extend the investigations, 8 fractions (numbered 1 to 8; Figure 1) were collected from the

Figure 4. Reducing and nonreducing SDS-PAGE of copolymers isolated from lactoplasma proteins of the pressurized milk P500 (500 MPa/55°C) by anion-exchange fast protein liquid chromatography. Volumes of 10 μ L (copolymers at 2 mg/mL) were loaded in the gel. M = mixture of molecular mass standards reduced by 2-mercaptoethanol; Lf = lactoferrin.

FPLC separation of the P500 lactosera proteins and identified by alkaline PAGE (Figure 5a). "Native-like" α -LA was mainly recovered in fraction 4. The glycosylated forms of α -LA were eluted in fractions 3 and 4 (sialylated and nonsialylated glycoforms; Slangen and Visser, 1999). The other electrophoretic bands revealed by Schiff's reagent after periodic acid oxidation corres-

ponded to residual IgG (mainly recovered in fractions 4 and 5) and to component-3 of proteose peptone eluted in fractions 6 to 8. Glycosylated materials in fraction 8 that did not migrate into the gel might correspond to lactosylated copolymers or polymers of glycosylated κ -CN. These materials were, however, in very low quantities in P500 as well as P600 (Figure 1).

Two major bands (noted *a* and *b*; Figures 3 and 5) present in fractions 6 to 8 were not revealed by Schiff's reagent. They did not correspond to glycoforms of α -LA or to the N-glycosylated amino-terminal fragment 54–135 of component-3 released by endogenous plasmin in milk (Sørensen and Petersen, 1993). Band *a* was sensitive to the presence of the reducing agent, whereas band *b* was not sensitive and remained on the SDS-PAGE patterns independently of the presence or absence of 2-mercaptoethanol (Figures 3 and 5b). Their apparent molecular masses were approximately 19 and 22 kDa for bands *a* and *b*, respectively. Band *a* was found in all the samples, contrary to band *b* (under nonreducing conditions). Band *a* corresponded to β -LG, which alkyl thiol forms a cysteinyl-S-propionamide adduct by reacting with the double bond of free, unreacted acrylamide in gel (Chiari et al., 1992). Band *b* was generated only after drastic treatments and was recovered in lactosera of P500 and P600, specifically. Its absence in lactoplasma suggested that band *b* might interact with the CN micelles. After electrotransfer of reducing SDS-PAGE gel, band *b* was amino-terminal microsequenced and identified to amino-terminal fragments of β -CN (Arg-Glu-Leu-Glu-Leu-Asn-Val-Pro-) released in lactosera of P500 and P600.

DISCUSSION

The combination of pressure and heat induced different kinetics of protein aggregation (polymerization and copolymerization) than heat alone. Oligomerization of β -LG into "SDS-dimeric" and "SDS-trimeric" forms (termed by Havea et al., 2001) is usually observed by nonreducing SDS-PAGE of heated β -LG (Bauer et al., 1998; Schokker et al., 2000; Havea et al., 2001; Cho et al., 2003), but was not found in pressurized milk (Figure 3a). β -LG remained soluble (or in suspension) in lactoplasma after processing at 300 MPa/4°C or 400 MPa/20°C but had undergone polymerization and copolymerization with α -LA, minor whey proteins, and κ -CN via disulfide bond exchange, and to a lesser extent, with α _{s1}-CN through physicochemical interactions at 500 MPa/55°C or 600 MPa/70°C. The large copolymers formed displayed 2 groups of sizes, one group with molecular masses between 440 and 2000 kDa and a second group with molecular masses greater than 2000 kDa. In our conditions, the mechanism of aggregation of β -LG in

Table 1. Electrophoretic band areas (expressed as absorbance unit per mm) of the main lactosoluble and lactoplasma proteins of raw milk (control) and pressurized milk samples P300 (300 MPa/4°C), P400 (400 MPa/20°C), P500 (500 MPa, 55°C), and P600 (600 MPa/70°C), and α -LA-to- β -LG ratios determined by densitometry.

	Nonreducing SDS-PAGE			Reducing SDS-PAGE		
	α -LA	β -LG	α -LA/ β -LG	α -LA	β -LG	α -LA/ β -LG
Lactosoluble						
Control	1.9	3.5	0.6	1.8	3.4	0.5
P300	2.0	3.6	0.5	1.9	3.7	0.5
P400	2.2	3.6	0.6	1.9	3.5	0.5
P500	2.2	0.6	3.8	2.5	2.4	1.1
P600	2.1	0.4	6.0	2.1	1.8	1.2
Lactoplasma						
Control	2.1	3.6	0.6	1.7	3.4	0.5
P300	2.1	2.9	0.7	1.4	2.7	0.5
P400	2.2	3.4	0.7	1.4	2.6	0.5
P500	1.1	0.4	3.1	1.4	2.5	0.5
P600	1.1	0.3	3.4	1.3	2.0	0.6

milk would be faster than the polymerization that had been determined at 78.5°C with isolated β -LG (Schokker et al., 1999). Indeed, the absence of dimers and intermediate small oligomers suggested that the formation of reactive β -LG monomers (corresponding to the initiation step of the β -LG polymerization described by Roefs and De Kruif, 1994) might be very fast. The formation of copolymers, mainly composed of β -LG and κ -CN, would be controlled by the ratio of reactive β -LG to monomeric and polymeric κ -CN in milk (Farrell et al., 1998), but also by the kinetics of the formation of β -LG polymers. This formation might correspond to the early stages of milk gelation, as has already been stated for aggregation of heat-treated β -LG (Schokker et al., 1999). In another work, pressurization at 400 MPa applied to concentrated milk at 20°C for 10 min is sufficient to induce gelation (Famelart et al., 1998).

Under our conditions of pressure, temperature, and treatment time, no significant CN release was observed. However, it is worth noting that a β -CN amino-terminal fragment with an apparent molecular mass of 22 kDa was only recovered in the whey of milk samples treated at 500 MPa/55°C and 600 MPa/70°C. This fragment was different from β -CN-5P (f1-105 or f1-107 fragment), a peptide generated by plasmin in raw milk and also called component-5 of proteose peptone (apparent molecular mass of 18 kDa; Girardet et al., 1991). The 22-kDa peptide was similar to peptide P1 (similar apparent molecular masses and identical amino-terminal sequences) generated in milk by elastase, a predominant enzyme in PMNL (Verdi and Barbano, 1991), during a lipopolysaccharide experimental mastitis (Moussaoui et al., 2003). The degranulation of PMNL is the principal origin of the proteolytic activities other than plasmin. Mainly neutral proteases such as elastase, MMP-9, cathepsin G, and proteinase 3 have been found in

milk samples having a high SCC of more than 400,000 to 600,000 cells/mL (for review, see Le Roux et al., 2003). The SCC of the raw milk studied seemed to be high enough (309,000 cells/mL) to release proteolytic activities detectable in milk samples pressurized at 500 or 600 MPa and at 55 or 70°C, respectively. On the other hand, plasmin is known to be pressure-stable, retaining almost all activity even after treatment at 600 MPa for 20 min at 20°C (Scollard et al., 2000). However, despite its barostability, pressure combined with temperatures higher than 20°C (i.e., 40 to 60°C) increased plasmin inactivation (Garcia-Risco et al., 2000, 2003). In addition to possible release of elastase from the PMNL into the milk's soluble phase, alteration of CN micelles by combination of pressure and temperature might enhance the susceptibility of CN to proteolysis by increasing either the micelle surface area available or by the exposure of new substrate sites on proteins (Ohmiya et al., 1989; Shibauchi et al., 1992).

CONCLUSIONS

Synergistic effects of pressure and temperature on raw milk resulted in the formation of large copolymers initiated by a very fast polymerization of reactive β -LG monomers. Lactosylation of β -LG and α -LA that usually occurs in heat-treated milk samples seemed to be very limited, as only trace amounts of glycosylated aggregates (corresponding to Maillard reaction's products or polymers of κ -CN) were found. Proteolysis was not significantly increased by milk treatment, although limited hydrolysis of β -CN occurred in pressurized and heated milk samples. More drastic treatments might affect the barostability of milk leading to gel formation.

Figure 5. Alkaline PAGE (A) and reducing SDS-PAGE (B) of lactosoluble proteins of raw milk (control C), of the pressurized milk samples P500 (500 MPa/55°C) and P600 (600 MPa/70°C), and of chromatographic fractions 1 to 8 obtained from lactosoluble proteins of P500. Volumes of 20 μ L (proteins at 2 mg/mL) were loaded in the gels. Bands *a* and *b* corresponded to cysteinyl-S-propionamide derivative of β -LG and amino-terminal fragment of β -CN, respectively. M = mixture of molecular mass standards; Lf = lactoferrin; PP3 = proteose peptone component-3. (+) bands stained by Schiff's reagent after periodic acid oxidation; (*) sialylated and (**) nonsialylated N-glycosylated α -LA.

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REFERENCES

- Bauer, R., S. Hansen, and L. Ogendal. 1998. Detection of intermediate oligomers, important for the formation of heat aggregates of β -lactoglobulin. *Int. Dairy J.* 8:105–112.
- Chiari, M., P. G. Righetti, A. Negri, F. Cecilian, and S. Ronchi. 1992. Preincubation with cysteine prevents modification of sulfhydryl groups in proteins by unreacted acrylamide in a gel. *Electrophoresis* 11:882–884.
- Cho, Y., H. Singh, and L. K. Creamer. 2003. Heat-induced interactions of β -lactoglobulin A and κ -casein B in a model system. *J. Dairy Res.* 70:61–71.
- Davies, D. T., and A. J. R. Law. 1983. Variation in the protein composition of bovine casein micelles and serum casein in relation to micellar size and milk temperature. *J. Dairy Res.* 50:67–75.
- Egito, A. S., J.-M. Girardet, L. Miclo, and J.-L. Gaillard. 2001. Highly sensitive periodic acid/Schiff detection of bovine milk glycoproteins electrotransferred after nondenaturing electrophoresis, urea electrophoresis, and isoelectric focusing. *Lait* 81:775–785.
- Famelart, M.-H., F. Gaucheron, F. Mariette, Y. Le Graet, K. Raulot, and E. Boyaval. 1997. Acidification of pressure-treated milk. *Int. Dairy J.* 7:325–330.
- Famelart, M.-H., L. Chapron, M. Piot, G. Brulé, and C. Durier. 1998. High pressure-induced gel formation of milk and whey concentrates. *J. Food Eng.* 36:149–164.
- Farrell, H. M., Jr., E. D. Wickham, and M. L. Groves. 1998. Environmental influences on purified κ -casein: Disulfide interactions. *J. Dairy Sci.* 81:2974–2984.
- Funtenberger, S., E. Dumay, and J. C. Cheftel. 1995. Pressure-induced aggregation of β -lactoglobulin in pH 7.0 buffers. *Lebensm. Wiss. Technol.* 28:410–418.
- Funtenberger, S., E. Dumay, and J. C. Cheftel. 1997. High pressure promotes β -lactoglobulin aggregation through SH/S-S interchange reactions. *J. Agric. Food Chem.* 45:912–921.
- Garcia-Risco, M. R., A. Olano, M. Ramos, and R. Lopez-Fandino. 2000. Micellar changes induced by high pressure. Influence in the proteolytic activity and organoleptic properties of milk. *J. Dairy Sci.* 83:2184–2189.
- Garcia-Risco, M. R., I. Recio, E. Molina, and R. Lopez-Fandino. 2003. Plasmin activity in pressurized milk. *J. Dairy Sci.* 86:728–734.
- Girardet, J.-M., A. Mati, T. Sanogo, L. Etienne, and G. Linden. 1991. Fast protein liquid chromatography of hydrophobic fraction of bovine milk proteose-peptone and characterization by bidimensional electrophoresis. *J. Dairy Res.* 58:85–98.
- Havea, P., H. Singh, L. K. Creamer, and O. H. Campanella. 1998. Electrophoretic characterization of the protein products formed during heat treatment of whey protein concentrate solutions. *J. Dairy Res.* 65:79–91.
- Havea, P., H. Singh, and L. K. Creamer. 2001. Characterization of heat-induced aggregates of β -lactoglobulin, α -lactalbumin and bovine serum albumin in a whey protein concentrate. *J. Dairy Res.* 68:483–497.
- Iametti, S., P. Transidico, F. Bonomi, G. Vecchio, P. Pittia, P. Rovere, and G. Dall'Aglio. 1997. Molecular modifications of β -Lactoglobulin upon exposure to high pressure. *J. Agric. Food Chem.* 45:23–29.
- Kapitany, R. A., and E. J. Zebrowski. 1973. A high resolution PAS stain for polyacrylamide gel electrophoresis. *Anal. Biochem.* 65:361–369.

- Laemmli, U. K., and M. Favre. 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J. Mol. Biol.* 80:893–900.
- Le Roux, Y., F. Laurent, and F. Moussaoui. 2003. Polymorphonuclear proteolytic activity and milk composition change. *Vet. Res.* 34:629–645.
- Lopez-Fandino, R., A. V. Carrascosa, and A. Olano. 1996. The effects of high pressure on whey protein denaturation and cheese-making properties of raw milk. *J. Dairy Sci.* 79:929–936.
- Moussaoui, F., F. Laurent, J.-M. Girardet, G. Humbert, J.-L. Gaillard, and Y. Le Roux. 2003. Characterization and proteolytic origins of specific peptides appearing during lipopolysaccharide experimental mastitis. *J. Dairy Sci.* 86:1163–1170.
- Ohmiya, K., T. Kajino, S. Shimizu, and K. Gekko. 1989. Dissociation and reassociation of enzyme-treated caseins under high pressure. *J. Dairy Res.* 56:435–442.
- Pâquet, D., Y. Nejjar, and G. Lindon. 1988. Study of hydrophobic protein fraction isolated from milk proteose-peptone. *J. Dairy Sci.* 71:1464–1471.
- Roefs, S. P. F. M., and K. G. De Kruif. 1994. A model for the denaturation and aggregation of β -lactoglobulin. *Eur. J. Biochem.* 226:883–889.
- Schokker, E. P., H. Singh, D. N. Pinder, G. E. Norris, and L. K. Creamer. 1999. Characterization of intermediates formed during heat-induced aggregation of β -lactoglobulin AB at neutral pH. *Int. Dairy J.* 9:791–800.
- Schokker, E. P., H. Singh, and L. K. Creamer. 2000. Heat-induced aggregation of β -lactoglobulin A and B with α -lactalbumin. *Int. Dairy J.* 10:843–853.
- Scollard, P. G., T. P. Beresford, E. C. Needs, P. M. Murphy, and A. L. Kelly. 2000. Plasmin activity, β -lactoglobulin denaturation and proteolysis in high pressure treated milk. *Int. Dairy J.* 10:835–841.
- Shibauchi, Y., H. Yamamoto, and Y. Sagara. 1992. Conformational change of casein micelles by high pressure treatment. Pages 239–241 in *High Pressure and Biotechnology*. C. Balny, R. Hayashi, K. Heremans, and P. Masson, eds. John Libbey Eurotext Ltd., Montrouge, France.
- Slangen, C. J., and S. Visser. 1999. Use of mass spectrometry to rapidly characterize the heterogeneity of bovine α -lactalbumin. *J. Agric. Food Chem.* 47:4549–4556.
- Sørensen, E. S., and T. E. Petersen. 1993. Phosphorylation, glycosylation and amino acid sequence of component PP3 from the proteose-peptone fraction of bovine milk. *J. Dairy Res.* 60:535–542.
- Tedford, L. A., and C. J. Schaschke. 2000. Induced structural change to β -lactoglobulin by combined pressure and temperature. *Biochem. Eng. J.* 5:73–76.
- Verdi, R. J., and D. M. Barbano. 1991. Properties of protease from milk somatic cells and blood leukocytes. *J. Dairy Sci.* 74:2077–2081.