Rennet Coagulation of Milk Subjected to High Pressures

Rosina López-Fandiño,* Mercedes Ramos, and Agustín Olano

Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

The rennet coagulation properties of highly pressurized milk have been investigated. The initial rate and extent of the enzymatic phase of coagulation, determined on the basis of the release of caseinomacropeptides (CMP) soluble in 4% trichloroacetic acid, were inhibited by pressures over 200 MPa. However, the coagulation time decreased as pressure increased up to 200 MPa and then increased again, until at 400 MPa, it reached values comparable with those of the raw milk. Therefore, the coagulation time of the pressurized milk was not directly related to the degree of κ-casein hydrolysis, indicating that pressure probably favored the aggregation phase. Addition of $CaCl_2$ enhanced casein aggregation of native or pressurized milk, reducing coagulation time and curd-firming time, but did not offset the rate-increasing (up to 200 MPa) or rate-lowering (at 300 and 400 MPa) effects of milk pressurization on subsequent casein coagulation by rennet. The SDS−PAGE studies of pressure-treated and untreated milk or solutions containing κ-casein, β-lactoglobulin, or both in the presence or absence of denaturing agents showed evidence for the formation of aggregates linked by intermolecular disulfide bonds.

Keywords: *High pressure; rennet coagulation; protein aggregation*

INTRODUCTION

High-pressure treatment of foodstuffs is a rapidly growing preservation technology that, even if in the development stage, presents the potential for making products with better functional and microbiological properties (Cheftel, 1992). Though high-pressure food processing is currently a focus of major interest (Schmidt and Buchheim, 1970; Ohmiya et al., 1987; Johnston et al., 1992; Shibauchi et al., 1992; Desobry-Banon et al., 1994), many of the effects of pressure on milk components are still not known.

We previously reported that treatment at pressures up to 400 MPa improved the cheese yield and coagulation properties of milk (López-Fandiño et al., 1996). Pressurization under 200 MPa decreased the rennet coagulation time and increased the curd-firming rate. However, the coagulation time of milk pressurized over 200 MPa increased and the curd-firming rate decreased, until, at 400 MPa, they reached values comparable with those of the raw milk. In addition, treatment of milk at pressures over 100 MPa progressively increased the denaturation of β-lactoglobulin (β-Lg).

Thermal denaturation of β-Lg is known to affect the cheese-making properties of milk. It has been claimed that heating of milk affects the clotting process by slowing or inhibiting the primary phase of rennet action, because κ-casein−β-Lg cross-linking reduces the susceptibility of κ-casein to hydrolysis by chymosin (Leaver et al., 1995; van Hooydonk et al., 1987). In addition, the complex formed between whey proteins and caseins may interfere with the aggregation stage, following the enzymatic hydrolysis of κ-casein, presumably because of steric hindrance of the β-Lg that is bound to the surface of the micelles (Marshall, 1982; van Hooydonk et al., 1987).

The main objective of this study was to investigate the effect of pressures up to 400 MPa on the enzymatic hydrolysis of κ-casein and the subsequent aggregation of casein micelles, to explain the renneting behavior of pressurized milk. The existence of pressure-induced associations between β-Lg and κ-casein was also investigated.

* Author to whom correspondence should be addressed (tel, +34 1 5622900; fax, +34 1 5644853; e-mail, ifilf34@fresno.csic.es).

MATERIALS AND METHODS

High-Pressure Processing. Raw bovine milk was obtained from a local dairy farm and kept refrigerated for a maximum of 3 h before use. Samples of 300 mL were poured into polyethylene bottles, a lid was placed avoiding headspace, and the bottles were vacuum-sealed in polyethylene bags before being pressurized.

Solutions of β-Lg, κ-casein, or mixtures of β-Lg and κ-casein (Sigma Chemical Co., St. Louis, MO) were prepared in 50 mM Tris-HCl buffer, pH 6.8, at a concentration of 2.5 mg/mL of each protein. In previous experiments, no significant effects of pressures under 500 MPa on milk pH were observed (López-Fandiño et al., 1996; Johnston et al., 1992); therefore, a pressure-resistant buffer was chosen to ensure that pH would be constant under high pressure (Funtenberger et al., 1995). Each solution (2.5 mL) was then pressurized in small polyethylene tubes.

Samples were pressurized using a 900 HP apparatus (Eurotherm Automation, Lyon, France). The pressure was raised to the desired value (100−400 MPa) at a rate of 2.5 MPa/s, maintained for 10−60 min, and finally released at the same rate. The temperature of the hydrostatic fluid medium was measured with a thermocouple, and before pressure processing, it was controlled at 25 ± 1 °C by circulating water through a jacket surrounding the pressure vessel. High-pressure treatments were performed in triplicate with milk from different batches or freshly prepared protein solutions. Samples were kept refrigerated at 5 °C and analyzed within 20 h after pressurization. All the analytical determinations were performed at least in triplicate.

Primary Phase of Rennin Action. The extent of the enzymatic reaction was determined on the basis of the release of caseinomacropeptide (CMP) soluble in 4% trichloroacetic acid (TCA), as analyzed by reversed-phase (RP) HPLC. To milk (30 mL) that had been prewarmed at 30 °C was added 750 μL of a 0.04% (w/v) crystalline chymosin solution (EC 3.2.23.4, activity approximately 23.6 units/mg of protein; Sigma Chemical Co.). This concentration of coagulant was lower than that used to determine the rennet coagulation

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properties of milk, because a slow release of CMP was necessary to accurately determine the initial rate of the enzymatic phase of coagulation. Samples were incubated at 30 °C, and 2-mL aliquots were withdrawn from the reaction mixtures at intervals. The reaction was stopped by vigorously vortex-mixing into 4 mL of 6% (w/v) TCA. In the case of the pure protein solutions, 1800 μL was treated with 44 μL of 0.04% (w/v) crystalline chymosin solution at 30 °C and, at appropriate time intervals, 200-μL aliquots were mixed as just described with 400 μL of 6% TCA. After 30 min at 30 °C, the samples were centrifuged (4500*g* for 20 min at 5 °C), filtered through a Durapore 0.45-μm filter (Millipore Corp., Bedford, MA), and analyzed by RP-HPLC.

A Beckman System Gold HPLC instrument was used together with a System Gold Software data acquisition system (Beckman Instruments Inc., San Ramon, CA). Separations were performed on a Beckman Ultrapore RPSC column (75 × 4.6 mm) as described previously (López-Fandiño et al., 1993). Solvent A was 0.1% trifluoroacetic acid (TFA; Merk, Darmstadt, Germany) in Milli-Q water (Millipore Corp.), and solvent B was 0.1% TFA in acetonitrile (HPLC grade; Scharlau, Barcelona, Spain). A flow rate of 1 mL/min was employed with detection at 210 nm.

Coagulation Properties of Milk. A Formagraph (N. Foss and Co., Hillerod, Denmark) was used to measure rennet coagulation properties: coagulation time (*r*), curd-firming time (k_{20}), and curd firmness (a_{30}) (McMahon and Brown, 1982). The coagulation properties of raw and pressurized milks with and without $CaCl_2$ added were determined. To milk, was added $CaCl_2$ to a concentration of 4 mM, and the mixture was left to equilibrate for 30 min at 30 °C. Milk pH did not change because of the addition of $CaCl_2$, and so it was not readjusted. To measure the rennet clotting properties, 200 μL of standard rennet (85% chymosin and 15% bovine pepsin, strength 1:10000; Chr. Hansen's Lab., Copenhagen, Denmark) solution (0.3%, w/v) in acetate buffer (0.2 M, pH 5.4) was added to 10 mL of milk that had been preequilibrated to 36 °C for 30 min. The coagulation properties of the samples with a lower amount of coagulant, used to determine the primary phase of rennet action, were also determined.

Analysis of Caseins and Whey Proteins. Whey proteins and caseins were separated from raw and pressurized milk samples after the pH was adjusted to 4.6 with 2 M HCl.

Denaturation of β-Lg following the pressure treatments was determined by quantitative analyses of native β-Lg soluble at pH 4.6 and is expressed as percentage of the β-Lg content of the corresponding raw milk. Whey proteins that were soluble at pH 4.6 were determined by RP-HPLC (Resmini et al., 1989). Separations were performed on a PLRP 8-μm column (300 Å, 150 × 4.6 mm; Polymer Laboratories, Church Stretton, U.K.) with a linear binary gradient. Solvent A was 0.1% (w/v) TFA (Pierce, Rockford, IL) in HPLC grade water (LabScan Ltd., Dublin, Ireland), and solvent B consisted of 0.1% (w/v) TFA in acetonitrile. The wavelength was set at 205 nm.

Lyophilized caseins or protein solutions were dissolved either in 10 mM Tris-HCl buffer, pH 8.0 (nonreducing conditions), or in the same buffer containing 2.5% SDS, 10 mM EDTA, and 5.0% β-mercaptoethanol (reducing conditions) and heated at 100 °C for 10 min. Analysis by SDS−PAGE used the PhastSystem electrophoresis apparatus, precast PhastGels homogeneous 20%, and PhastGel SDS buffer strips (Pharmacia, Uppsala, Sweden). Electrophoretic conditions and staining with PhastGel blue R followed the procedures of the manufacturer.

Following SDS−PAGE under nonreducing conditions, protein bands were immediately transferred onto 0.45-μm nitrocellulose paper (Bio-Rad, Richmond, CA) by a 10-min diffusion step. The transfer and immunodetection of the antigens were performed as described by Molina et al. (1996). A commercial polyclonal antiserum raised in rabbit anti-bovine β-Lg (Nordic Immunology, Tilburg, The Netherlands) was used.

Statistical Analysis. One-way ANOVA of the data was carried out by using the Statgraphics statistical system (Statgraphics, 1991). The data employed corresponded to three independent experiments with three analytical determinations made for each set.

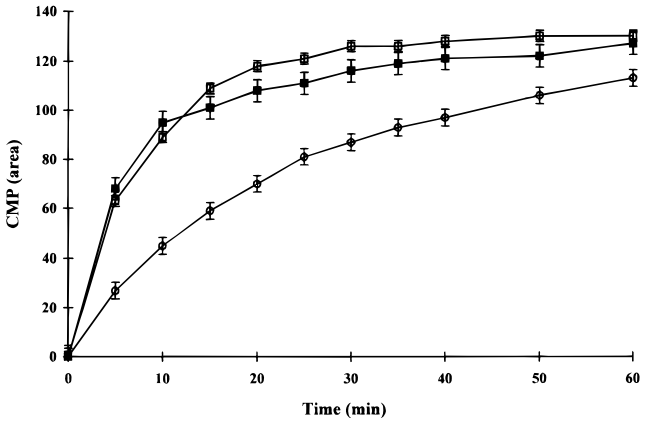

Figure 1. Release of CMP from raw milk (□) and milk pressurized at 200 (■) and 400 (○) MPa for 30 min and incubated with chymosin at 30 °C. The means ± the standard errors of three independent experiments are shown.

RESULTS AND DISCUSSION

Effects of Pressure Treatment on the Primary Phase of Renneting. Figure 1 shows the CMP release, as a function of time, from raw milk and from milk pressurized at 200 and 400 MPa for 30 min. As previously mentioned, the amount of coagulant used for these experiments was lower than that normally used in cheese making and led to coagulation times of 27, 17, and 27 min for raw milk and milks pressurized at 200 and 400 MPa, respectively. Pressurization of milk at 200 MPa for 30 min hardly slowed the enzymatic reaction, although, in agreement with previous results (López-Fandiño et al., 1996), pressurization denatured about 20% of the β-Lg and reduced the coagulation time as determined with the Formagraph (which comprises both enzymatic and aggregation phases) by 37% with respect to the raw milk. When milk was pressurized to 400 MPa for 30 min, the rate of formation and the final level of CMP decreased (Figure 1), showing that the enzymatic stage of the coagulation process was inhibited by prior pressure processing of milk. However, although denaturation of β-Lg reached 90%, the coagulation time was similar to that of the raw milk. van Hooydonk et al. (1987) found that the primary phase of coagulation was almost unaffected in heat-treated milk that had undergone denaturation of 25% of the whey proteins. Those authors reported, however, a significant delay in the aggregation phase. No coagulation was observed in the case of heat-treated milk samples showing denaturation of 75% of the whey proteins (van Hooydonk et al., 1987).

The effect of a previous high-pressure treatment on the hydrolysis of κ-casein with chymosin was confirmed in pressurized solutions of pure proteins (Figure 2). Treatment of mixtures of β-Lg and κ-casein at 400 MPa for 30 min, in a pressure-resistant buffer, reduced the susceptibility of κ-casein to be hydrolyzed by chymosin, while when κ-casein was pressurized in the absence of β-Lg, the release of CMP was at the same rate as from unpressurized κ-casein. This suggests that the attachment of β-Lg to κ-casein under pressure restricts the subsequent hydrolysis of κ-casein by chymosin.

Effect of $CaCl_2$ Addition. Table 1 shows the influence of the addition of 4 mM $CaCl_2$ on the coagulation properties of raw and pressurized milk. The effects of the pressure treatments on the subsequent coagulation of milk in the absence of $CaCl_2$ have already been studied, and the present results agree with previously

Table 1. Coagulation Properties (Means (SE) of Three Independent Experiments) of Raw Milk and Milk Treated with Different Pressures for 30 min with and without Addition of 4 mM $CaCl_2$[a]

pressure (MPa)	r (min)		k_{20} (min)		a_{30} (mm)	
	without $CaCl_2$	with 4 mM $CaCl_2$	without $CaCl_2$	with 4 mM $CaCl_2$	without $CaCl_2$	with 4 mM $CaCl_2$
0	13.9^{a} (0.3)	5.5^{b,c} (0.1)	10.3^{a} (0.2)	4.5^{a} (0.3)	27.3^{c} (0.3)	27.0^{c} (0.6)
100	10.3^{c} (0.2)	5.0^{c,d} (0.1)	7.0^{b} (0.3)	3.2^{c} (0.2)	30.2^{b} (0.9)	26.7^{c} (0.3)
200	8.7^{d} (0.1)	4.5^{d} (0.3)	5.2^{c} (0.1)	2.7^{c} (0.2)	29.8^{b} (1.3)	26.7^{c} (0.3)
300	11.3^{b} (0.2)	5.7^{b} (0.2)	6.2^{b} (0.2)	3.8^{b} (0.2)	41.0^{a} (0.7)	35.3^{b} (0.9)
400	14.2^{a} (0.2)	6.8^{a} (0.2)	9.5^{a} (0.5)	4.8^{a} (0.2)	30.8^{b} (1.0)	39.7^{a} (0.9)

[a] Means in the same column followed by different superscripts (a−d) differ ($P < 0.05$).

Table 2. Coagulation Properties (Means (SE) of Three Independent Experiments) of Raw Milk and Milk Treated at 400 MPa for Different Periods with and without Addition of 4 mM $CaCl_2$[a]

time (min)	r (min)		k_{20} (min)		a_{30} (mm)	
	without $CaCl_2$	with 4 mM $CaCl_2$	without $CaCl_2$	with 4 mM $CaCl_2$	without $CaCl_2$	with 4 mM $CaCl_2$
0	13.9^{b,c} (0.3)	5.5^{d} (0.1)	10.3^{b} (0.2)	4.5^{b,c} (0.3)	27.3^{c} (0.3)	27.0^{c} (0.6)
10	11.8^{d} (0.2)	6.1^{c} (0.2)	7.2^{d} (0.3)	4.0^{c} (0.3)	39.6^{a} (0.7)	45.7^{a} (1.6)
20	13.3^{c} (0.3)	7.0^{b} (0.1)	8.8^{c} (0.3)	4.7^{b,c} (0.2)	34.0^{b} (1.0)	42.3^{b} (0.7)
30	14.2^{b} (0.2)	6.8^{b} (0.2)	9.5^{b,c} (0.5)	4.8^{b} (0.2)	30.8^{c} (1.0)	39.7^{b} (0.9)
60	18.8^{a} (0.4)	8.2^{a} (0.2)	14.5^{a} (0.3)	6.5^{a} (0.3)	16.6^{d} (0.9)	41.0^{b} (0.6)

[a] Means in the same column followed by different superscripts (a−d) differ ($P < 0.05$).

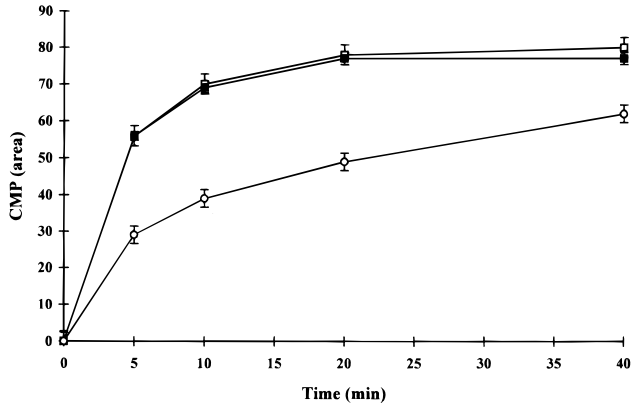

Figure 2. Release of CMP from solutions of κ-casein (□), κ-casein pressurized at 400 MPa for 30 min (■), and the mixture of κ-casein and β-lactoglobulin pressurized at 400 MPa for 30 min (○) and incubated with chymosin at 30 °C. The means ± the standard errors of three independent experiments are shown.

published data (López-Fandiño et al., 1996). The addition of $CaCl_2$ reduced the coagulation time (r) and curd-firming time (k_{20}) of raw milk by 60% and 56%, respectively. When $CaCl_2$ was added to pressure-treated samples, the coagulation times (r) were reduced by approximately 50% as compared to the same samples without the addition of the salt. Because the curd-firming time (k_{20}) depends on the coagulation time, the reductions observed in k_{20} when $CaCl_2$ was added were also less pronounced than those in raw milk. The addition of $CaCl_2$ did not significantly change the curd firmness (a_{30}) of the raw milk but led to slightly weaker curds in the case of milks treated at 100, 200, and 300 MPa, as compared with the same samples without the addition of the salt. However, the curd firmness of milk pressurized at 400 MPa was significantly improved by the addition of $CaCl_2$.

Addition of Ca^{2+} to milk accelerates the overall clotting process because of the effect on the aggregation stage of the reaction (Lucey and Fox, 1993). Addition of $CaCl_2$ stimulates coagulation, curd-firming rate, and whey loss, probably because $CaCl_2$ binds to casein micelles reducing the repulsive forces and promoting hydrophobic interactions (Green, 1982), and can reverse the adverse effects of heating milk on subsequent coagulation (Singh et al., 1988; van Hooydonk et al., 1987). However, present results show that, although the addition of $CaCl_2$ reduced the coagulation time and increased the curd-firming rate of pressurized milk, the effect is not so marked as with raw milk. Changes in the salt distribution as a consequence of pressure treatments have not been so thoroughly studied as those caused by heating. Schmidt and Buchheim (1992) reported that pressurization of milk caused micelle disintegration, which was reversible on adding calcium salts. This reduction in micellar size can enhance coagulation (Ford and Grandison, 1986). But although it was found that calcium was released on milk pressurization and this was also related to the fragmentation of the micelle structure (Shibauchi et al., 1992; Desobry-Banon et al., 1994), Johnston et al. (1992) did not find any changes in the ionic Ca^{2+} content of milk after pressure treatment.

The effect of the addition of $CaCl_2$ on the coagulation properties of milk pressurized at 400 MPa for different times is illustrated in Table 2. When $CaCl_2$ was added, the coagulation times of milks treated at 400 MPa were significantly longer than that of the raw milk (with $CaCl_2$). The curd firmness (a_{30}) of milks pressurized at 400 MPa was much more increased by $CaCl_2$ than the a_{30} of the native milk. However, in agreement with previous results (López-Fandiño et al., 1996), when no $CaCl_2$ was added, the highest a_{30} was achieved after 10 min and was followed by a rapid decrease. Unfortunately, with the present knowledge, it is difficult to explain many of the observed effects of high pressure on milk. Further experiments will need to be conducted in order to understand the present results.

Complex Formation between β-Lg and κ-Casein in Pressurized Milk. The effects of pressure on aggregation of β-Lg and κ-casein were studied by SDS−PAGE under nonreducing (Figure 3a) and reducing (Figure 3b) conditions. Under nonreducing conditions, few qualitative differences were found between the protein precipitated at pH 4.6 of milk pressurized at 400 MPa for 30 min and that of raw milk (Figure 3a, lanes 7 and 8), except there seemed to be more material of molecular weight higher than 80 kDa in the pressurized samples. The band corresponding to monomer κ-casein was absent, and a band with an apparent molecular mass of about 80 kDa, as well as another component

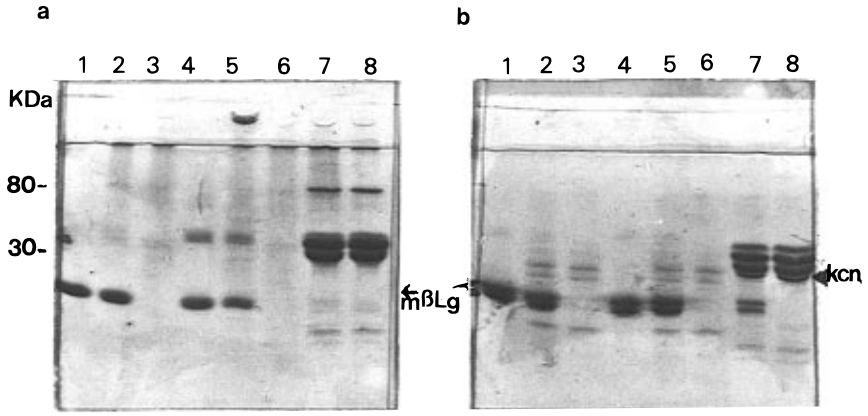

Figure 3. SDS−PAGE under nonreducing (a) and reducing (b) conditions: lane 1, β-Lg; lane 2, a mixture of κ-casein and β-Lg; lane 3, κ-casein; lane 4, β-Lg pressurized at 400 MPa for 30 min; lane 5, a mixture of κ-casein and β-Lg pressurized at 400 MPa for 30 min; lane 6, κ-casein pressurized at 400 MPa for 30 min; lane 7, protein precipitated at pH 4.6 from milk pressurized at 400 MPa for 30 min; lane 8, protein precipitated at pH 4.6 from raw milk. mβ-Lg = monomeric β-Lg; kcn = κ-casein.

located in the boundary between the stacking and resolving gels, was present in both samples. Upon the addition of β-mercaptoethanol (Figure 3b, lanes 7 and 8), the high molecular mass bands disappeared, the band corresponding to κ-casein became noticeable, and two bands, probably corresponding to fully and partially reduced β-Lg (Hurley et al., 1993), appeared clearly in the electrophoregram of the pressure-treated sample, suggesting that disulfide bonds were at least partially responsible for the intermolecular interactions of β-Lg.

To clarify whether those pressure-induced interactions involved β-Lg alone or whether an association with κ-casein was also being formed, model systems of β-Lg, κ-casein, and β-Lg plus κ-casein were treated in a pressure-resistant buffer (Funtenberger at al., 1995) at 400 MPa for 30 min. Pressurization of β-Lg alone (Figure 3a, lane 4) led to the formation of aggregates of apparent molecular weight of over 30 kDa, as well as to other, fainter bands, with apparent molecular masses between 40 and 70 kDa. These aggregates seemed to be completely solubilized when the electrophoresis was conducted in the presence of a reducing agent (Figure 3b, lane 4). It should be noted that under nonreducing conditions, monomeric β-Lg migrates at a lower apparent molecular mass than under reducing conditions (Hurley, 1993). Funtenberger et al. (1995) studied the pressure-induced aggregation of β-Lg in various buffers at pH 7.0 and reported the progressive formation of dimers and trimers (38−42 kDa), tetramers (69 kDa), pentamers (100 kDa), and higher polymers, which also disappeared on treatment with β-mercaptoethanol.

Pressurized mixtures of β-Lg and κ-casein, in the absence of β-mercaptoethanol (Figure 3a, lane 5), gave patterns very similar to those of β-Lg alone (Figure 3a, lane 4), suggesting that most of the aggregates are formed independently of the presence of κ-casein. The only difference was a band, of presumably very high molecular mass, which would not leave the stacking gel to enter the resolving gel. This band disappeared from the SDS−PAGE pattern when the sample was treated with 2-mercaptoethanol (Figure 3b, lane 5) and, therefore, could be a disulfide-linked complex of β-Lg and κ-casein.

Immunoblotting of proteins separated under nonreducing conditions increased the sensitivity of detection of β-Lg in casein obtained from pressurized milk (Figure 4, lane 7) and in mixtures of β-Lg and κ-casein (Figure 4, lane 5). Comparison of those patterns with the pattern of pressurized β-Lg alone (Figure 4, lane 4) did

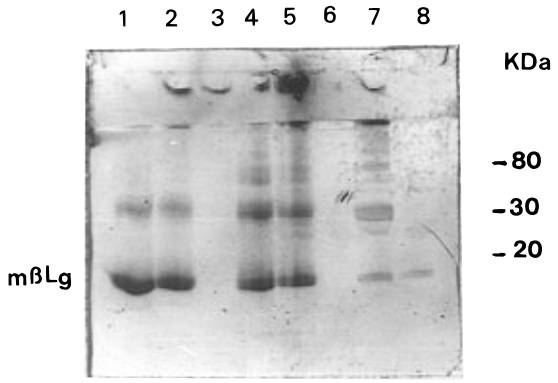

Figure 4. Immunoblot with antiserum against β-Lg of a SDS−PAGE plate under nonreducing conditions: lane 1, β-Lg; lane 2, a mixture of κ-casein and β-Lg; lane 3, κ-casein; lane 4, β-Lg pressurized at 400 MPa for 30 min; lane 5, a mixture of κ-casein and β-Lg pressurized at 400 MPa for 30 min; lane 6, κ-casein pressurized at 400 MPa for 30 min; lane 7, protein precipitated at pH 4.6 from milk pressurized at 400 MPa for 30 min; lane 8, protein precipitated at pH 4.6 from raw milk. mβ-Lg = monomeric β-Lg.

not show noticeable differences; the only exception was the high molecular weight complex that appeared very strongly when β-Lg and κ-casein were treated together. Low molecular mass aggregates (dimers and trimers) were already present both in samples of β-Lg and in mixtures of β-Lg and κ-casein before pressurization.

These results might indicate either that pressure-induced aggregation of β-Lg predominates over its association with casein or that the complexes formed were too large to be detected by gel electrophoresis without reducing agents. However, it is also possible that not only denatured β-Lg molecules but also β-Lg aggregates would associate further with κ-casein, giving rise to high molecular mass compounds that would be indistinguishable by this method from others containing β-Lg exclusively.

CONCLUSIONS

The present results indicate that, although the enzymatic phase of coagulation is inhibited by previous pressure processing of milk, the coagulating behavior of highly pressurized milk cannot be directly related to the degree of κ-casein hydrolysis. It is likely, therefore, that the effects of pressure on the aggregation phase play a major role. Thus, the overall reduction in micellar size that is induced by pressure (Schmidt and

Buchheim, 1970; Desobry-Banon et al., 1994) could favor the stages of curd making that depend on casein aggregation (Ford and Grandison, 1986), since this size reduction would increase the casein surface area available for interaction and promote cross-linking. Addition of $CaCl_2$ enhanced casein aggregation of native or pressurized milk, reducing coagulation time and curd-firming time, but did not offset the rate-increasing (up to 200 MPa) or rate-lowering (at 300 and 400 MPa) effects of milk pressurization on subsequent casein coagulation by rennet. Nevertheless $CaCl_2$ significantly increased the curd firmness of milks pressurized at 400 MPa. Denaturation of β-Lg, occurring during pressure treatments over 100 MPa, and its association with κ-casein could not only impair the enzymatic reaction but also hinder the aggregation of renneted micelles, counteracting the effects derived from the reduction in micellar size and explaining that after processing at 300 and 400 MPa coagulation rates are reduced. However the presence in pressurized milk of complexes of κ-casein and β-Lg could not be demonstrated.

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