The Effects of High Pressure on Whey Protein Denaturation and Cheese-Making Properties of Raw Milk

R. LOPEZ-FANDIÑO, A. V. CARRASCOSA, and A. OLANO
Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

ABSTRACT

The effects of high pressure, from 100 to 400 MPa (14,500 to 58,000 psi) and applied for different periods (10 to 60 min), on the biochemical and microbiological characteristics of milk have been investigated. Particular attention was paid to modifications of the cheese-making properties. High pressure slightly improved the microbiological quality of milk without modifying the activity of native milk enzymes such as lactoperoxidase or plasmin. β-Lactoglobulin was denatured by pressures >100 MPa, but α-lactalbumin and bovine serum albumin were resistant to pressures ≤400 MPa for 60 min. Cheese yield, as estimated by centrifugation of curd, increased with pressurization at 300 and 400 MPa, reflecting incorporation of additional β-lactoglobulin and, especially, greater moisture retention. The coagulation time decreased as pressure increased ≤200 MPa and then increased again until at 400 MPa, reaching values comparable with those of the raw milk. High pressure treatment of milk can be an effective method for improving the coagulation characteristics of milk and for increasing the moisture retention of fresh cheese with a minimum of modification of other properties important for cheese making.

(**Key words**: high pressure, cheese yield, milk coagulation)

INTRODUCTION

The application of high pressure to processing of foodstuffs is currently a subject of major interest for both food preservation and food preparation. Interest has increased because recent progress in the engineering of large-scale, high pressure equipment has allowed this technology to be adapted to the needs of the food industry.

Since the work of Hite (8), a number of papers have appeared on the preservation of milk, and considerable information is now available on various aspects of high pressure inactivation of microbes (24). In addition to microbial inhibition and destruction, milk constituents undergo physicochemical changes that lead to modifications in milk quality and functionality. Thus, several researchers have reported changes in the functional properties of caseins (20), which have often been observed to enhance milk coagulation (6) and improve gel rigidity and water-holding capacity (11). In many cases, such studies have been carried out using reconstituted NDM. Furthermore, microbiological studies have often relied on model systems that have been inoculated with pure bacterial strains. As a result, few data are currently available on the effects of high pressure treatments on the indigenous microbial flora and constituents of raw milks.

The aim of this work was to provide a study of biochemical and microbiological changes of raw milk upon exposure to high pressures from 100 to 400 MPa (14,500 to 58,000 psi) for different periods (10 to 60 min). Particular attention has been paid to the effects of pressure on the cheese-making properties, as exemplified by the rennet-clotting ability, and the final cheese yield.

MATERIALS AND METHODS

High Pressure Processing

Raw bovine milk was obtained from a local dairy farm and was kept refrigerated a maximum of 3 h before use. Samples of 300 ml were poured into polyethylene bottles, avoiding any headspace, and vacuum-sealed in polyethylene bags before being pressurized.

Milk was pressurized using a 900 HP apparatus (Eurotherm Automation, Lyon, France). The pressure was raised to the desired value (100 to 400 MPa) at a rate of 2.5 MPa/s, then maintained at this value for 10 to 60 min, and finally released at the same rate. The temperature of the sample was controlled by circulating water at 25 ± 1°C through a jacket surrounding the pressure vessel. High pressure treatments were performed in quadruplicate, and replicates were conducted on different days with milk from different batches. Samples were kept refrigerated at 5°C and were analyzed within 20 h after pressurization.

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Microbiological Analysis

Appropriate dilutions of raw and treated milk were made using sterile saline water (0.85%), and 0.1 ml were spread-plated on solid plate count agar (Oxoid, Basingstoke, Hampshire, England). Total aerobic and psychrotrophic counts were obtained after incubation at 30°C for 48 h and 7°C for 10 d, respectively. Colony diameters were estimated with a Haloes caliper (IUL Instruments GmbH, Konigswinter, Germany).

Analytical Determinations

Undenatured whey proteins were determined by reverse-phase HPLC as described by Resmini et al. (25). Whey proteins were separated from whole milk samples after the pH was adjusted to 4.6 with 2 *M* HCl. Separations were performed on a PLRP 8-μm column (300 Å, 150 × 4.6 mm i.d.; Polymer Laboratories, Church Stretton, England) with a linear binary gradient. Solvent A was 0.1% (wt/vol) trifluoroacetic acid (Pierce, Rockford, IL) in HPLC grade water (LabScan Ltd., Dublin, Ireland), and solvent B consisted of 0.1% (wt/vol) trifluoroacetic acid in acetonitrile (HPLC grade; LabScan Ltd.). The wavelength was set at 205 nm. A standard curve for β-lactoglobulin (Sigma Chemical Co., St. Louis, MO) was used for calibration.

Lactulose was determined as its trimethylsilyl derivative by GLC, using phenyl-β-D-glucoside (Sigma Chemical Co.) as the internal standard (23). Furosine was determined by ion-pair reverse-phase HPLC (5).

Alkaline phosphatase activity was determined qualitatively by the method of Aschaffenburg and Mullen (3). Lactoperoxidase activity was measured following a modification of the spectrophotometric method described by Shindler et al. (27): milk (25 μl) was added to 5 ml of 1 m*M* ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid; Sigma Chemical Co.) in 0.1 *M* acetate buffer at pH 4.4. Proteolytic activity was estimated after incubation of raw and pressure-treated milk samples for 48 h at 37°C. Milk was mixed with three preservatives: 10 m*M* NaN_3, 0.1 ml/ml $CHCl_3$, and 0.1 ml/ml of toluene (2). The fraction that was soluble at pH 4.6 was obtained by adjusting the pH with 2 *M* HCl, followed by centrifugation (4500 × *g* for 15 min at 5°C), and then filtration through Whatman number 49 filter paper (Whatman Corp., Clifton, NJ). The proteolytic activity was calculated as the difference in percentage of nitrogen that was soluble at pH 4.6, as determined by the Kjeldahl method, before and after incubation. Alternatively, the hydrolysates were also analyzed by reverse-phase HPLC as described previously (12).

A Formagraph (N. Foss and Co., Hellerup, Denmark) was used to measure rennet coagulation properties: coagulation time (r), the time when the curd is firm enough for cutting (k_{20}), and curd firmness (a_{30}) following the method described by McMahon and Brown (15). In this case, 200 μl of a standard rennet (85% chymosin and 15% bovine pepsin, strength 1:10,000, Chr. Hansen's Lab, Copenhagen, Denmark) solution (0.3 g/100 ml) in acetate buffer (0.2 *M*, pH 5.4) were added to 10 ml of milk.

Cheese yields were estimated by centrifugation, which provided a good assessment of fresh cheese yield unadjusted for moisture content (13). Milk (30 ml), prewarmed to 30°C, was treated with 600 μl of rennet solution (identical to that used for the coagulation properties measurements) and stirred for 1 min. After 40 min at 30°C, the curd was cut and, 10 min later, centrifuged at 15,000 × *g* for 15 min at a temperature of 5°C. The curd and whey were then separated; the nitrogen in the whey was determined by the Kjeldahl method and expressed as total nitrogen per 100 ml of milk by considering the total volume of whey drained.

All the analytical determinations were performed at least in triplicate.

Statistical Analysis

One-way ANOVA of the data was carried out by using the Statgraphics Statistical System (29). The data employed corresponded to four independent experiments with three analytical determinations made for each set.

RESULTS

Effects of High Pressure on Microbiological and Chemical Changes of Milk

The effects of pressures from 100 to 400 MPa, for 30 min, on the total aerobic and pyschrotrophic bacterial counts are shown in Figure 1. Although the lethal effect increased as pressure increased, the total and psychrotrophic counts only differed statistically from the initial counts for treatments >100 MPa ($P < 0.01$). Numbers of psychrotrophic bacteria were reduced with pressure more quickly than total bacterial counts. At 300 MPa, no psychrotrophic bacteria were detected by the plate count method (<10 cfu/ml). However, this failure in growth initiation could have been due to the lower incubation temperature; these microorganisms might have regenerated under more favorable conditions. Under certain circumstances, high pressure might not inactivate cells but might instead injure a proportion of the population (24), and evidence of damaged cells was suggested by

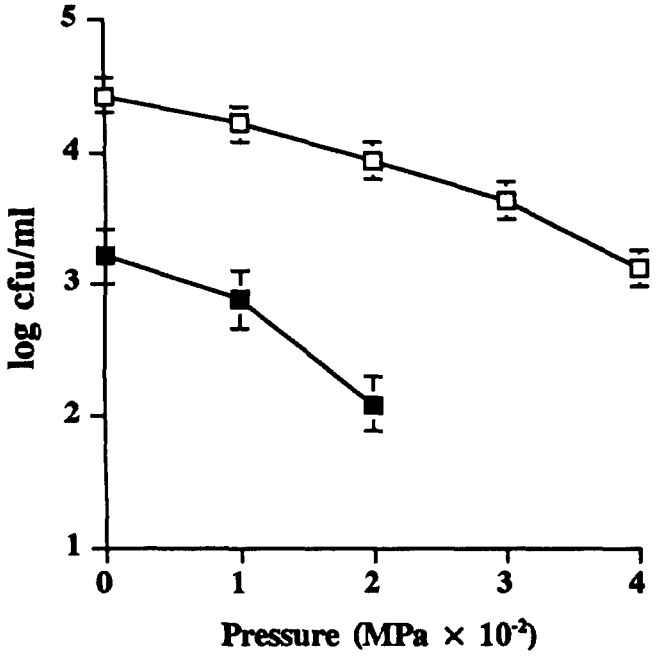

Figure 1. Effect of pressure after 30 min on total aerobic (□) and psychrotrophic (■) counts of milk. The means (±SE) of four independent experiments are shown.

the morphology of the colonies. More than 75% of the colonies of bacteria recovered from fresh milk had diameters >0.5 mm, and 100% of the colonies of total bacteria population recovered from milk submitted to 400 MPa and psychrotrophic bacteria from milk treated at 200 MPa had diameters <0.5 mm.

The effects of exposure time at 200 and 400 MPa are shown in Figure 2. At 200 MPa, the total bacterial count decreased slightly as time increased, but the psychrotrophic count was markedly reduced during the first 20 min of treatment. At 400 MPa, the curve for total bacteria leveled off after 10 min, with no further increase in the lethal effect. Patterson et al. (24) reported that the pressure-mediated death curves often show survival tails, which have been related, among other factors, to population heterogeneity. No psychrotrophic bacteria were detected after 10 min at 400 MPa (<10 cfu/ml). After 60 min at 200 MPa and 20 min at 400 MPa, 50 and 100%, respectively, of the colonies on plate count agar had diameters <0.5 mm.

The reduction in bacterial numbers induced by pressure treatment was in accordance with previous research on fresh (31) and contaminated (30) milk samples. Total bacterial population and psychrotrophic bacteria were less sensitive to pressure than were several food pathogens, such as *Salmonella* (16) or *Vibrio parahemolyticus* (30) treated in buffers.

High pressure microbial inactivation was not accompanied by chemical changes similar to those

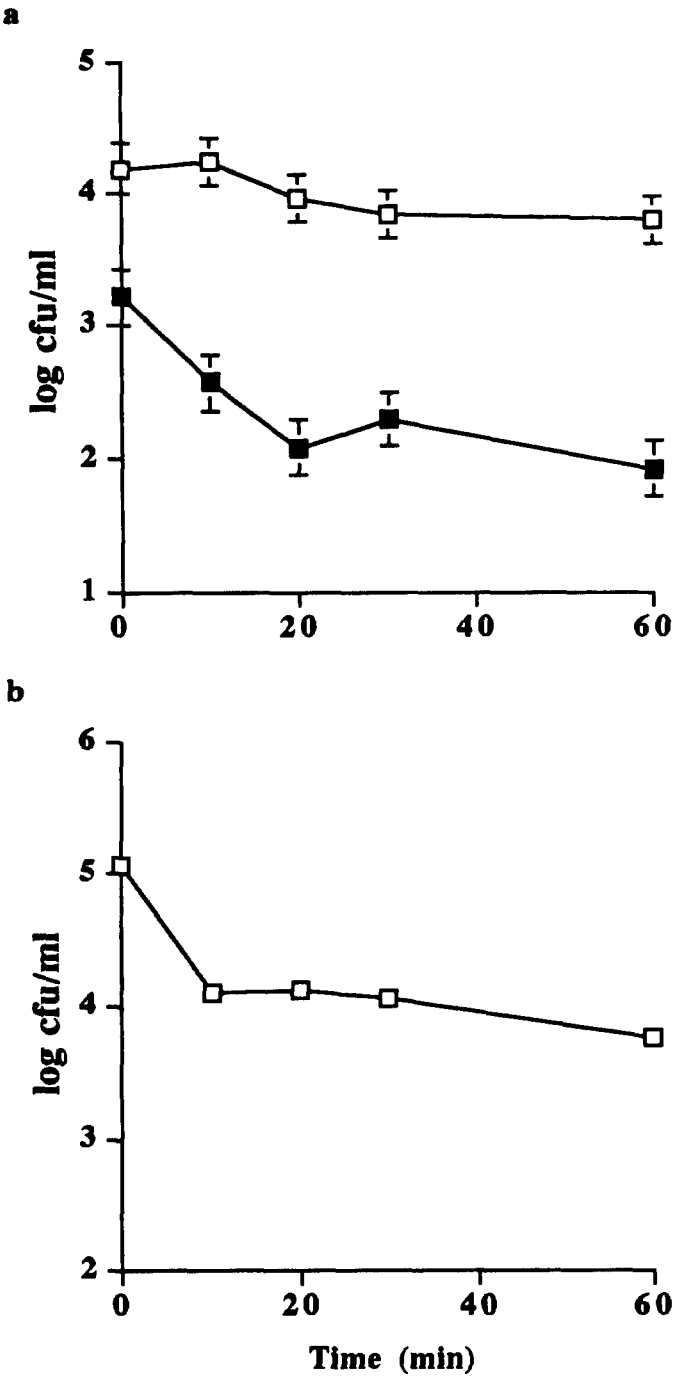

Figure 2. Effect of 200 (a) and 400 MPa (b) of pressure for various times on total aerobic (□) and psychrotrophic (■) counts of milk. The means (±SE) of four independent experiments are shown.

caused by treatments at high temperatures. No Maillard reaction or lactose isomerization occurred in any treated samples. Similarly, it was not possible to assess the process conditions by measuring the activity of the native enzymes that are indicators of heat load. Alkaline phosphatase, the basis for evaluating the effectiveness of pasteurization, was active in all the pressure-treated samples. Johnston (9) also reported that approximately 30% of the alkaline phosphatase activity was lost after 20 min at 400 MPa. Lactoperoxidase activity was not reduced, even after 60 min at 400 MPa.

The proteolytic activity of pressurized milk, as measured after 48 h of incubation at 37°C, was identical to that of the raw milk. The HPLC analyses of the hydrolysates (results not shown) showed that the main degradation products corresponded to the proteose-peptone component 5, which resulted from the action of plasmin on β-casein. The response of enzymes to pressures in the range of 100 to 400 MPa varied. Acid protease activity in beef was enhanced by 100 to 200 MPa of pressure and decreased slightly at 400 MPa, but neutral and alkaline protease activities remained almost constant at pressures up to 500 MPa (21).

No significant effect of pressure on milk pH was observed. Johnston et al. (10) reported that pH and calcium ion activity of milk were unaltered by pressures ≤600 MPa.

β-Lactoglobulin was denatured by pressures over 100 MPa, but α-lactalbumin and bovine serum albumin appeared to be completely resistant to pressures ≤400 MPa for 60 min. The resistance of α-lactalbumin to pressure can be attributed to the lack of free sulfhydryl groups and to its rigid molecular structure. Nakamura et al. (17) and Okamoto et al. (22) have reported that high pressures can be used in combination with proteolytic enzymes to digest selectively the β-lactoglobulin from whey protein concentrates, thereby reducing their antigenicity.

Effects of Pressure on Cheese Yield

The denaturation and resulting incorporation of whey proteins into the curd should effectively result in an increase in cheese yield, primarily by retention of moisture. Pressurization of milk for 30 min at 300 and 400 MPa caused estimated mean curd weight increases of 14 and 20%, respectively (Figure 3b) and a substantial decrease in protein loss in the whey fraction (7.5 and 15%, respectively; Figure 3c). Although pressurization of milk for 30 min at 200 MPa brought about a 20% denaturation of β-lactoglobulin (Figure 3a), the curd weight only increased significantly ($P < 0.01$) above this pressure. In addition to the greater retention of β-lactoglobulin in the curd, the total volume of whey also decreased by 4.5 and 5.8% in milk treated for 30 min at 300 and 400 MPa, respectively. Although the increase in curd weight reflected the incorporation of additional β-lactoglobulin, the increase was mainly because of a greater degree of moisture retention. Johnston et al. (11) found that acid-set gels prepared from pressurized skim milk showed less syneresis and improved water-binding characteristics with pressure ≤400 MPa.

The effect of holding time at a given pressure is illustrated in Figure 4. Curd weight and protein loss in the whey did not change with changes in exposure time at 200 MPa, even though β-lactoglobulin denaturation gradually increased to 42% over the treatment period. At 400 MPa, both the curd yield caused by moisture retention and protein recovery increased as the holding time increased, but the changes were greatest during the first 20 min of treatment. Nevertheless, a very rapid denaturation of β-lactoglobulin (amounting to 78%) was observed in the first 10 min of pressurization.

Effects of Pressure on Rennet Coagulation Properties of Milk

Figure 5 shows the coagulation properties of milk subjected to pressures of 100 to 400 MPa for 30 min as determined by the Formagraph. Coagulation time (r) and the time when the curd is firm enough for cutting (k_{20}) decreased as pressure increased up to 200 MPa, but increased as pressures were increased to 400 MPa to reach values that were not statistically different from those of the raw milk. This pressure dependence of clotting time was not in agreement with the results of others (6, 19, 26). Desobry-Banon et al. (6) reported that pressurization at ≥230 MPa enhanced coagulation of acid and rennet milk. However, those authors (6) used reconstituted low heat NDM precluding direct comparison with our results. Data on whole milk that were recently reported by Johnston (9) were similar to the present results.

Curd firmness at cutting (a_{30}), did not change significantly as pressure increased, except for milk treated at 300 MPa for 30 min, in which case curd firmness showed an intermediate maximum, followed by a decrease at 400 MPa. Desobry-Brown et al. (6) observed that pressurized milk obtained from low heat NDM produced stronger gels than did unpressurized milk. Curd firmness at cutting was considered

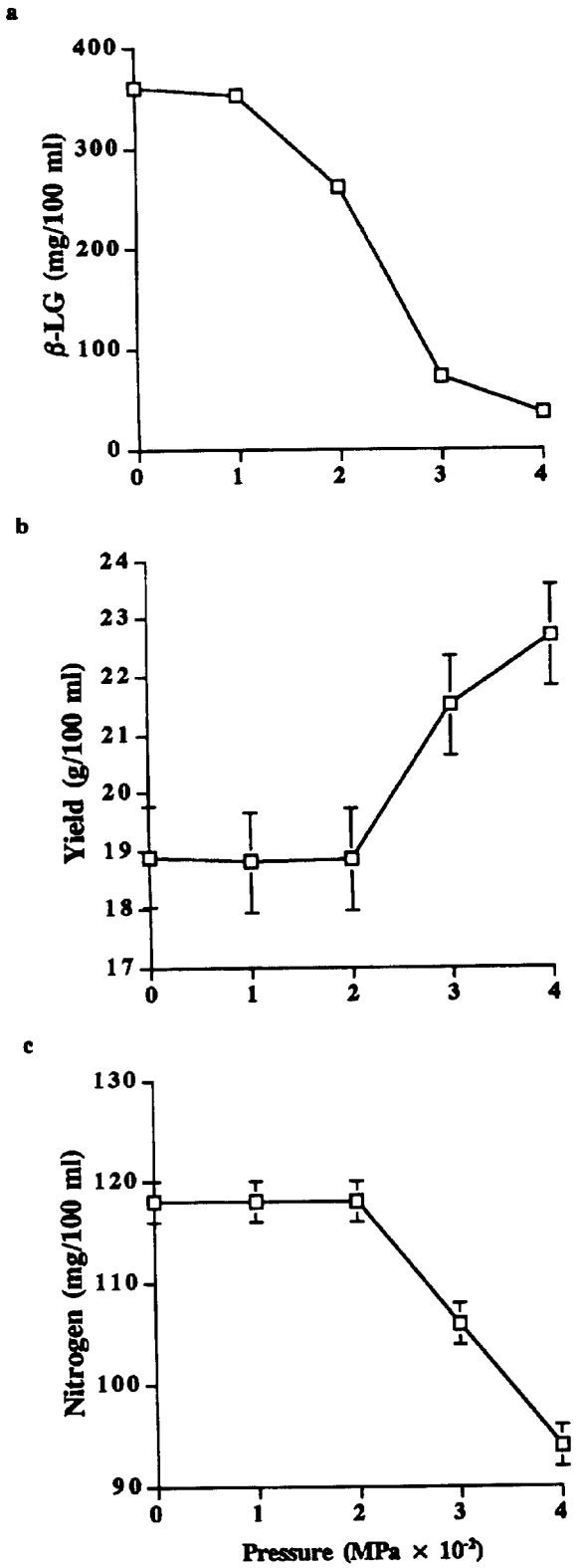

Figure 3. Effect of milk pressurization for 30 min on the concentration of native β-lactoglobulin (a), cheese yield as estimated by centrifugation (b), and nitrogen loss in the whey (c). The means (±SE) of four independent experiments are shown.

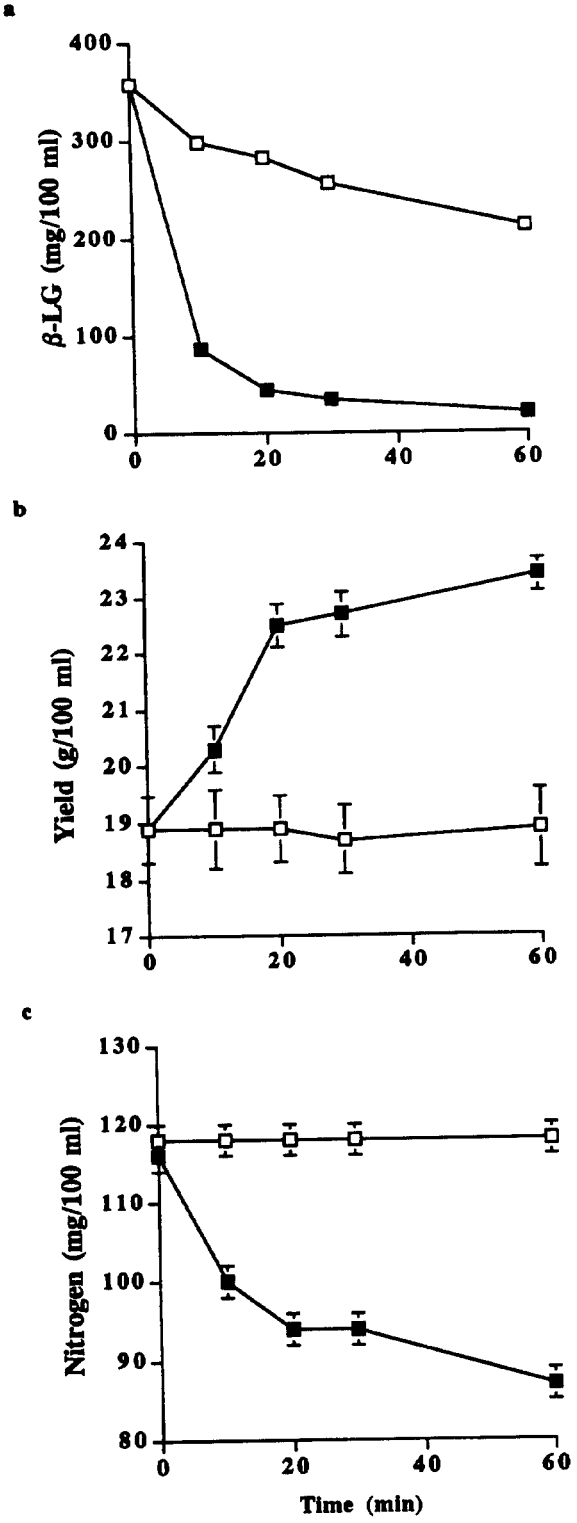

Figure 4. Effect of 200 (□) and 400 MPa (■) of pressure for different times on the concentration of native β-lactoglobulin in milk (a), cheese yield as estimated by centrifugation (b), and nitrogen loss in the whey (c). The means (±SE) of four independent experiments are shown.

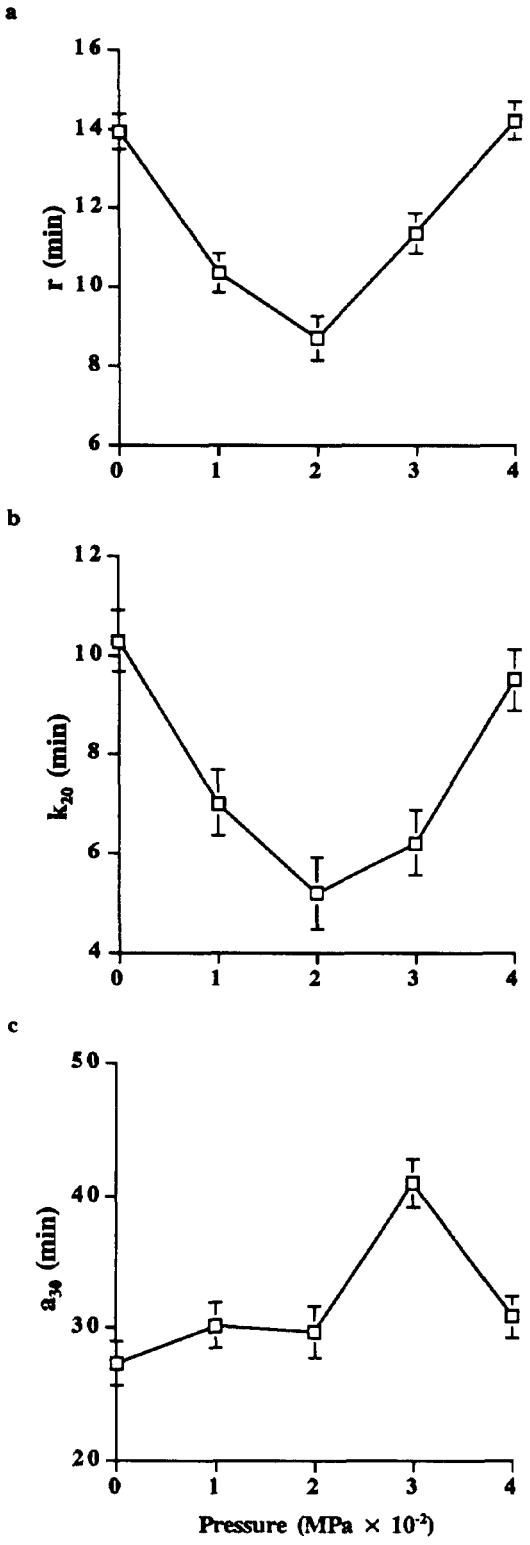

Figure 5. Effect of pressure after 30 min on the rennet clotting properties of milk: coagulation time (r) (a), time when the curd is firm enough for cutting (k_{20}) (b), and curd firmness (a_{30}) (c). The means (±SE) of four independent experiments are shown.

to be one of the main factors that influenced cheese yield (1).

Figure 6 illustrates the effect of the holding time at 200 and 400 MPa on the coagulation properties of milk. During the first 10 min of treatment at 200 MPa, coagulation time (r) and time when the curd is firm enough for cutting (k_{20}) decreased, and curd firmness (a_{30}) increased compared with those values for raw milk. Further exposure at 200 MPa of pressure did not cause changes in these parameters. At 400 MPa, coagulation time (r) and time when the curd is firm enough for cutting (k_{20}) decreased during the first 10 min of treatment and then subsequently increased up to 60 min. The curd firmness (a_{30}) of milk treated at 400 MPa was highest after 10 min and was followed by a rapid decrease.

DISCUSSION

This paper shows that treatment of whole milk with pressures from 100 to 300 MPa improved its coagulation characteristics; pressures of ≥300 MPa increased protein and moisture retention in the curd. In particular, treatment of milk at 300 MPa for 30 min decreased the coagulation time by 19% and increased the curd firming rate and the curd firmness by 39 and 58%, respectively, while causing an 80% denaturation of β-lactoglobulin and a 13% increase in curd weight, as estimated by centrifugation. High pressure could also be used to improve the microbial quality of milk, although the reductions obtained in the range of pressures applied in this study were not enough to guarantee product safety. However, the combined use of pressure and other treatments could be very useful in food preparation.

Among other methods, heat treatment has been used to promote whey protein denaturation and, consequently, increase its recovery in the curd (14). However, heating impaired the gel-forming properties of milk, led to longer coagulation times and weaker gels (32), and resulted in a practical limit of a 50% maximum of whey protein that could be incorporated (4). Unlike heat treatment, pressurization of milk to <400 MPa improved milk coagulation properties, although, at pressures >200 MPa, the rennet coagulation time increased as the pressure and time of exposure increased. Desobry-Banon et al. (6) found that the mean size of casein micelles decreased as the pressure increased from 230 to 430 MPa. This drop in particle size was accompanied by conformational changes from spherical particles into chains or clusters of submicelles (26) and could be responsible for the enhanced coagulation at 200 MPa, because of increased interparticle collision and reduced steric

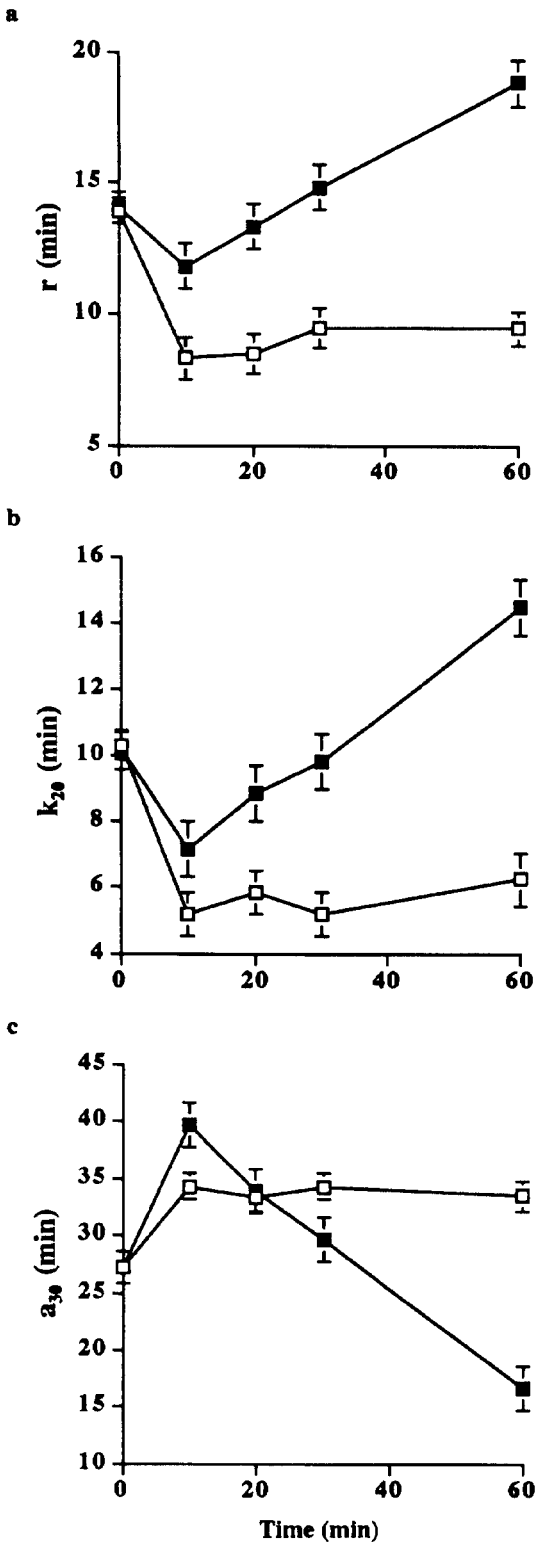

Figure 6. Effect of 200 (□) and 400 MPa (■) of pressure for various times on the rennet-clotting properties of milk: coagulation time (r) (a), time when the curd is firm enough for cutting (k_{20}) (b), and curd firmness (a_{30}) (c). The means (±SE) of four independent experiments are shown.

repulsion (7). However, the reason for the increase in rennet coagulation time of milk pressurized over 200 MPa remains unclear. Reduced casein micelle size has been associated with the production of firmer curds (18), although, in this case, increased curd firmness was found only at 300 MPa.

Although the potential interactions between β-lactoglobulin and caseins in pressurized milk are not known and may not be similar to those occurring in heated milk, the increased denaturation of β-lactoglobulin at ≥300 MPa could have counterbalanced the benefits brought about by the reduction in micellar size in a manner similar to its inhibitory effect on the rennet-clotting properties of heated milk. The lack of irreversible denaturation of α-lactalbumin, which is known to play a role in the gel-forming ability of heated mixtures (28), has to be taken into account. However, the increase in the rennet coagulation time at pressures >200 MPa could not be related directly to the degree of β-lactoglobulin denaturation, probably because of a distinct influence of pressure-induced protein denaturation on the primary and secondary phases of rennet coagulation.

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