Comparison of the Effects of High-Pressure Treatments and Heat Pasteurization on the Whey Proteins in Goat's Milk

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · MARCH 1997

Impact Factor: 2.91 · DOI: 10.1021/jf960406o

Comparison of the Effects of High-Pressure Treatments and Heat Pasteurization on the Whey Proteins in Goat's Milk

Xavier Felipe,[†] Marta Capellas,[†] and Andrew J. R. Law*[,‡]

Hannah Research Institute, Ayr KA6 5HL, Scotland, U.K., and Unitat de Tecnologie del Aliments, CeRTA, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

Goat's milk was subjected to pressures up to 500 MPa at 25 or 50 °C, and the extent of denaturation of the individual whey proteins, determined from their loss of solubility at pH 4.6, was examined by gel permeation FPLC (fast protein liquid chromatography) and SDS−PAGE. On pressure treatment at 25 °C, β-lactoglobulin readily aggregated, whereas the immunoglobulins and α-lactalbumin were more resistant. At 50 °C, the effect was greater, and the immunoglobulins and α-lactalbumin were also partially denatured. SDS−PAGE showed that after pressure treatment the proteins in the acid precipitate were disulfide linked. Some small soluble aggregates of β-lactoglobulin remained in the acid whey from pressurized milk, and these were not present in acid whey from heat-treated milk. Thermal and pressure treatments of milk could be distinguished by their different effects on denaturation of the individual whey proteins. Also, the activity of alkaline phosphatase in goat's milk was reduced by pasteurization but not by high-pressure treatment up to 500 MPa for 10 min. The possible use of these differences for monitoring thermal and pressure treatments in milk, and the importance of high pressure treatment in denaturation of the whey proteins, are discussed.

Keywords: *Whey protein; high pressure; goat's milk; indices; aggregation; denaturation*

INTRODUCTION

The effect of high pressure on food products has been studied since 1899 (Hite, 1899). However, it was only in the late 1980s that the technology evolved sufficiently for the process to be developed commercially. High-pressure equipment which was previously used for the production of metals, ceramics, and composites is now being considered for use in the food industry. Most of this progress has been made in Japan, where several pressurized foods such as jam and fruit juices have been available on the market since 1990 (Mertens, 1993). These treated products have longer shelf lives than the fresh foods and keep the same flavor and nutritive characteristics. Some studies have been carried out on the effect of high pressure on milk. Desobry-Banon *et al.* (1994) showed in their work on reconstituted milk that this treatment affects the rennet coagulation time, turbidity, and levels of soluble calcium and nitrogen. Johnston *et al.* (1992) found an increase in hydrophobic groups on the surface of the milk proteins and also noticed a decrease in non-casein nitrogen after treatment, indicating precipitation of the whey protein. Johnston *et al.* (1993) reported that there was an increase in water-holding capacity, protein hydration index, gel rigidity, and gel breaking strength of acid-set gels prepared from pressurized milk.

Pasteurization and microfiltration have been used to reduce the contaminant microflora in goat's milk (Gay *et al.*, 1993). Pasteurization is the main treatment used to reduce the growth of microorganisms in goat's milk but, unfortunately, also brings about undesirable changes in flavor and loss of nutrients. In this study, in order to determine the suitability of this new treatment for goat's milk, the effect of high pressure on denaturation of the whey proteins has been determined by following their loss of solubility at pH 4.6. Differences between pressure and thermal treatments in the extent of denaturation of the whey proteins and inactivation of alkaline phosphatase were also examined.

MATERIALS AND METHODS

Sample Preparation. Milk from Murciano-granadina goats was obtained from the farm of the Universitat Autònoma de Barcelona. The fat content was standardized to 4%, and the pH was adjusted to 6.7 with 1 M HCl or NaOH. Each milk sample for pressure treatment was placed in a tubular bag made of polyvinylidene chloride (31.8 mm in diameter, Krehalon, Soplaril Hispania S. A., Spain). The bags were vacuum packed to exclude air.

High-Pressure Treatment. The samples were treated using discontinuous hydrostatic high-pressure equipment (ACB, Nantes, France). The equipment reached 500 MPa in about 4 min and took between 90 and 120 s for pressure reduction. Treatment temperature was taken as the water temperature inside the cylinder, measured just before application of pressure. The pressure chamber and the water inside were cooled or heated to the required temperature by a constant flow of fluid within the walls of the vessel. Ten minutes before treatment started, the temperature of each sample was checked. After treatment the samples were refrigerated for 17 h before analysis or freezing was undertaken. The samples were pressurized at 25 or 50 °C, with a holding time of 5 or 10 min at the required pressure.

Heat Treatment. Skim-milk samples (5.0 mL) were heated in stoppered, thin-walled glass tubes (1 mm wall, 8 mm internal diameter, 160 mm length) in a waterbath. The milks were allowed 2 min 7 s to warm to 83 °C, maintained at this temperature for 1 min, and then rapidly cooled in ice.

Heat Pasteurization. Milk was pasteurized by passing through a heat-exchanger (Garvia S. A.) at 72 °C at a flow rate of 500 L/h, with a holding time of 15 s, and an overall time of 2 min 30 s for warming, holding, and cooling.

Acid Filtrate. Milk samples were warmed to 20 °C and adjusted to pH 4.6 with acetic acid (0.83 M) and sodium acetate

* Author to whom correspondence should be addressed.

[†] Universitat Autònoma de Barcelona

[‡] Hannah Research Institute.

S0021-8561(96)00406-2 CCC: $14.00

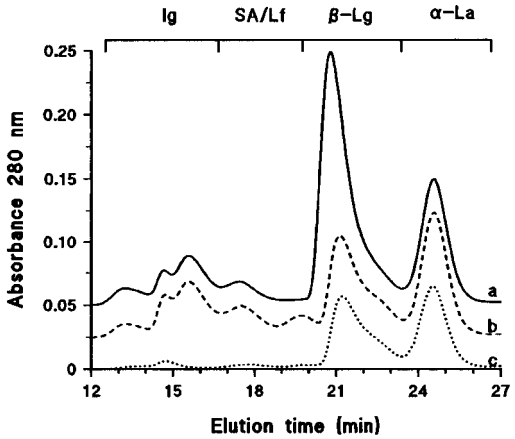

Figure 1. Elution profiles obtained by gel permeation FPLC on a Superdex 75 HR 10/30 column in Tris-HCl buffer (pH 7.0, 100 mM Tris, 0.5 M NaCl), showing the decrease in solubility of the whey proteins at pH 4.6 following heat and pressure treatments of goat's milk: (a) acid filtrate from raw milk; (b) acid filtrate from milk pressurized at 300 MPa for 5 min at 25 °C; (c) acid filtrate from milk heated at 83 °C for 1 min. Ig, immunoglobulins; SA/Lf, serum albumin and lactoferrin; β-Lg, β-lactoglobulin; α-La, α-lactalbumin.

(0.2 M), with a final dilution of 1:1. After allowing the samples to stand for 15 min, the supernatants were passed through 0.22 μm nylon filters (Sartorius, Epson, Surrey, U.K.).

Gel Permeation FPLC. Fast protein liquid chromatography (FPLC) was carried out on 50 μL aliquots of acid filtrate containing up to 3 mg of protein/mL. The samples were fractionated by gel permeation FPLC at 20 °C on a Superdex 75 HR 10/30 column (Pharmacia Biotech, St. Albans, U.K.) in Tris-HCl buffer (pH 7.0, 100 mM Tris, 0.5 M NaCl) at a flow rate of 0.5 mL/min. The absorbance of the eluate was monitored at 280 nm, and a total volume of 26 mL was passed through the column to ensure complete elution of absorbing material. Peak areas were corrected for the baseline value, and concentrations of the individual whey proteins calculated using specific absorbance coefficients at 280 nm: immunoglobulins, 12.1; serum albumin, lactoferrin, 6.9; β-lactoglobulin, 9.5; α-lactalbumin, 20.1 (Law and Brown, 1994).

SDS−PAGE. Whey proteins were examined by sodium dodecyl sulfate (SDS)−PAGE on PhastSystem electrophoresis equipment (Pharmacia Biotech, St. Albans, U.K.) using 20% homogeneous gels, in accordance with the manufacturers instructions.

Alkaline Phosphatase Activity. Activity was determined using a Fluorophos-R fluorometer according to Standard IDF Method 155 (1992). Each analysis was carried out on three different milks.

RESULTS

The elution profiles obtained by gel permeation FPLC of acid filtrates from goat's milk before and after thermal and pressure treatments are shown in Figure 1. The whey protein was separated into four fractions identified by Law and Brown (1994) as immunoglobulins (fraction 1), serum albumin and lactoferrin (fraction 2), β-lactoglobulin (fraction 3), and α-lactalbumin (fraction 4). Thermal treatment of the milk caused denaturation of the whey proteins and a decrease in the amount of whey proteins remaining in solution at pH 4.6. The fractions containing larger molecular weight proteins, such as the immunoglobulins and serum albumin/lactoferrin, were most easily denatured, whereas β-lactoglobulin was more heat-resistant, and α-lactalbumin was the most difficult to denature (Law, 1995). Following pressure treatment of milk at 25 °C, there was also a decrease in the amount of whey protein remaining in solution at pH 4.6 (Figure 1), indicating increased

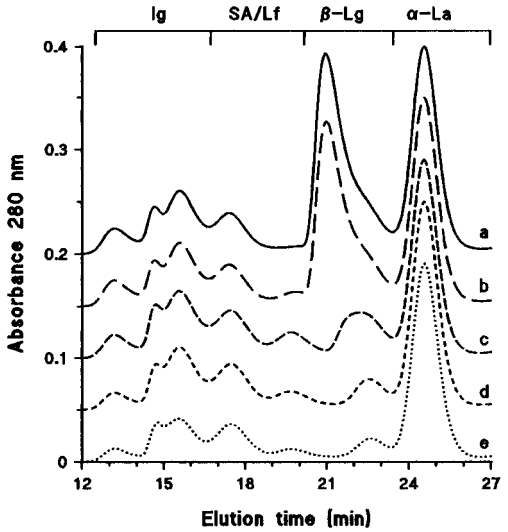

Figure 2. Elution profiles obtained by gel permeation FPLC (conditions as in Figure 1) showing the decrease in solubility of the whey proteins at pH 4.6 with increasing severity of pressure treatment of goat's milk at 25 °C: (a) acid filtrate from raw milk. Acid filtrates from milk pressurized for 10 min at (b) 200 MPa, (c) 300 MPa, (d) 400 MPa, and (e) 500 MPa. Abbreviations as in Figure 1.

aggregation of the protein. However, on pressure treatment, β-lactoglobulin was most easily denatured, and the fractions containing immunoglobulins, serum albumin/lactoferrin, and α-lactalbumin appeared to be much more resistant. As discussed below, there was also a small increase in the amount of lower molecular weight protein in fraction 2.

The effect of increasing the pressure up to 500 MPa at 25 °C on the whey protein fractions in milk is shown on Figure 2. At a pressure as low as 200 MPa, there was a decrease in the amount of β-lactoglobulin in the acid filtrate. None of the other fractions was affected, although a new peak appeared between the main peak of the serum albumin/lactoferrin fraction and β-lactoglobulin. On plotting the log of the molecular weight of the individual whey proteins against elution time (Law *et al.*, 1993), the molecular mass of the peak was estimated to be about 46 680 Da. The amount of this protein was greatest at about 300 MPa. Increasing pressure caused a decrease in the amount of this protein. At 300 MPa, the area of the serum albumin/lactoferrin fraction increased considerably, and there was also a slight increase in the region of the main fraction of immunoglobulins (IgG), but not in the minor fraction, which corresponded to the higher molecular weight IgM. At higher pressures (400 MPa), most of the β-lactoglobulin was denatured, and there was a slight decrease in the minor peak of IgM. Finally, at 500 MPa, there was some denaturation of the immunoglobulin fraction, but the serum albumin/lactoferrin and α-lactalbumin fractions did not show any appreciable decrease.

The effect of pressure treatment on the whey proteins in milk at 50 °C is shown in Figure 3. At 250 MPa, there was marked denaturation of β-lactoglobulin and the appearance of a small peak immediately before the β-lactoglobulin peak, but little change in the immunoglobulin and α-lactalbumin fractions. At 500 MPa, most of the β-lactoglobulin was denatured, and compared with treatments at 25 °C, there were higher levels of denaturation of the immunoglobulin and α-lactalbumin fractions.

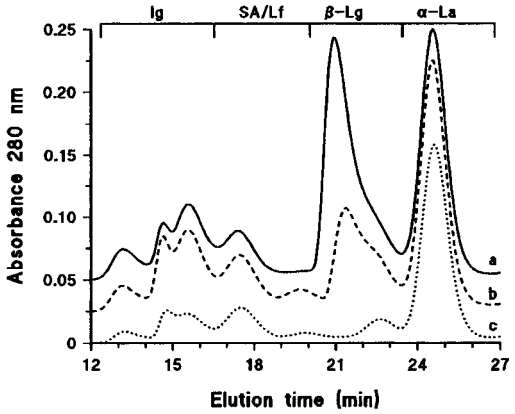

Figure 3. Elution profiles obtained by gel permeation FPLC (conditions as in Figure 1), showing the decrease in solubility of the whey proteins at pH 4.6 with increasing severity of pressure treatment of goat's milk at 50 °C: (a) acid filtrate from raw milk; (b) acid filtrate from milk pressurized at 250 MPa for 10 min; (c) filtrate from milk pressurized at 500 MPa for 10 min. Abbreviations as in Figure 1.

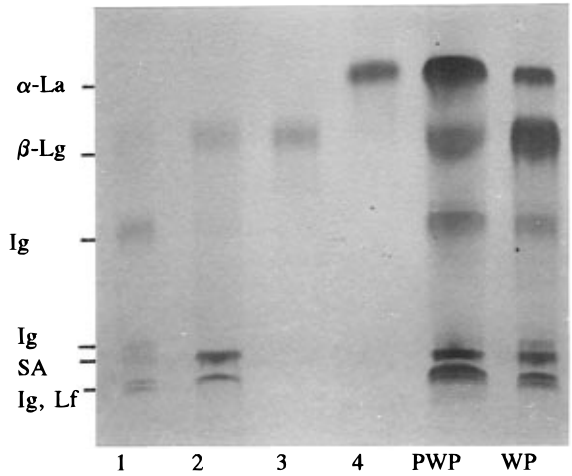

Figure 4. SDS−PAGE pattern on a 20% homogeneous gel for whole whey proteins (WP), whey proteins pressurized at 500 MPa for 10 min at 25 °C (PWP), and the fractions obtained by gel permeation FPLC as shown in Figure 2 of PWP (500 MPa for 10 min at 25 °C). Lanes 1−4, fractions 1−4, respectively. Abbreviations as in Figure 1.

The identities of the whey protein fractions obtained by gel permeation FPLC of acid filtrates from milks pressure treated at 500 MPa for 10 min at 25 °C were examined in more detail by SDS−PAGE (Figure 4). Under dissociating conditions, fraction 1 appeared to contain mainly immunoglobulins. Fraction 2 contained serum albumin and lactoferrin as major components, but a band of β-lactoglobulin was also clearly seen. Fraction 3 contained only β-lactoglobulin, and fraction 4 only α-lactalbumin. α-Lactalbumin was not detected in any of the other fractions. On similar pressure treatment at 50 °C (Figure 5), there was also evidence of the presence of some α-lactalbumin in the serum albumin/lactoferrin and β-lactoglobulin fractions. The presence of β-lactoglobulin in fraction 2, and of α-lactalbumin in fractions 2 and 3, was attributed to the formation of small aggregates which remained soluble at pH 4.6 and which could be disrupted in the presence of 2-mercaptoethanol.

Several studies have shown that when the whey proteins are aggregated by thermal treatment, they tend to precipitate at pH 4.6 together with the caseins (Rowland, 1937a,b). As can be seen in Figure 6, the acid precipitate from nontreated milk (lane 3r) contains very

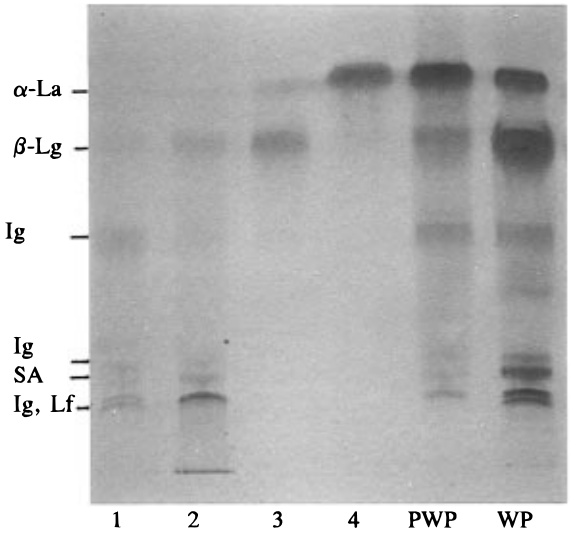

Figure 5. SDS−PAGE pattern on a 20% homogeneous gel for whole whey proteins (WP), whey proteins pressurized at 500 MPa for 10 min at 50 °C (PWP), and the fractions obtained by gel permeation FPLC as shown in Figure 3 of PWP (500 MPa for 10 min at 50 °C). Lanes 1−4, fractions 1−4, respectively. Abbreviations as in Figure 1.

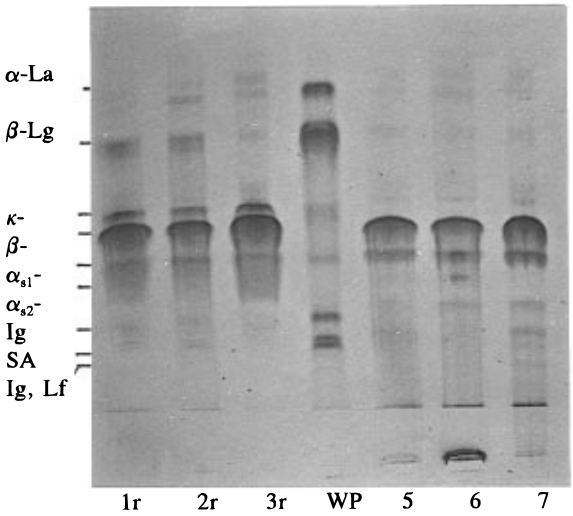

Figure 6. SDS−PAGE pattern on a 20% homogeneous gel for the proteins precipitated at pH 4.6. Lanes 1r and 5, acid precipitates from pressurized milk (500 MPa for 10 min at 25 °C); lanes 2r and 6, acid precipitates from heat-treated milk (83 °C for 1 min); lanes 3r and 7, acid precipitates from control milk; lane 4, whole whey proteins (WP). Lanes 1r, 2r, 3r, and 4, reduced with 2-mercaptoethanol. Lanes 5, 6, and 7, no reduction with 2-mercaptoethanol. α-La, α-lactalbumin; β-Lg, β-lactoglobulin, κ-, κ-casein; β-, β-casein; α_{s1}-, α_{s1}-casein; α_{s2}-, α_{s2}-casein; Ig, immunoglobulins; SA, serum albumin; Lf, lactoferrin.

little whey protein. However, the acid precipitates from pressurized milk (lane 1r), and heated milk (lane 2r) contain appreciable amounts of whey proteins. Following both treatments, most of the β-lactoglobulin precipitated at pH 4.6, but most of the α-lactalbumin was resistant to pressure and thermal treatments. However, there were differences in the behavior of both the immunoglobulins and serum albumin/lactoferrin following the two different types of treatment, with less of these proteins appearing in the acid precipitate following pressure treatment. On carrying out SDS−PAGE of the acid precipitated proteins from thermal- and pressure-treated milks without the reduction step with 2-mercaptoethanol (Figure 6), most of the whey proteins and κ-casein were absent from the running gel,

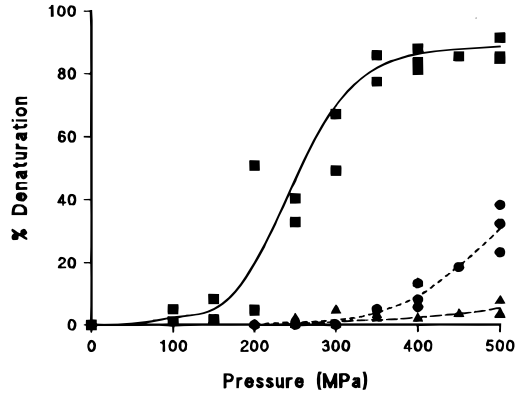

Figure 7. Effect of pressure at 25 °C on the extent of denaturation of each whey protein fraction, determined by gel permeation FPLC of acid filtrates from pressurized milk: (■) βLg, (●) Ig, (▲) αLa.

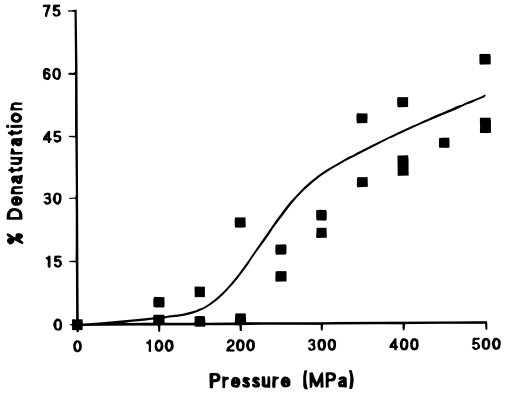

Figure 8. Effect of pressure at 25 °C on the extent of denaturation of total whey protein, determined by gel permeation FPLC of acid filtrates from pressurized milk.

and large aggregates appeared in the stacking gel, showing that these proteins were disulfide linked. The kind of aggregates could not be determined at this stage of the work, and they may be a mixture of homo- or heteropolymers.

The denaturation curves for the individual whey proteins on pressure-treatment of milk at 25 °C are shown in Figure 7. The serum albumin/lactoferrin fraction has not been plotted, as the presence of a small amount of β-lactoglobulin aggregates produced an increase in area following most of the treatments. However, previous studies on bovine serum albumin have indicated that it does not readily aggregate (Hayakawa *et al.*, 1992). The denaturation curve of β-lactoglobulin showed a slow increase up to 150−200 MPa, followed by a rapid increase up to 350 MPa. Above this pressure, most of the β-lactoglobulin was aggregated and the level of denaturation increased more slowly. Some variability in the levels of denaturation between milk samples for each treatment was observed, and this was related to the different initial concentrations of whey proteins. No differences could be observed in the levels of denaturation of the immunoglobulins with pressure treatments up to 300 MPa, but some aggregation occurred between 300 and 500 MPa. Results are similar to those of Howlett *et al.* (1992), who reported that bovine IgG does not undergo conformational changes below 210 MPa. They also found that when the pressure was increased to 820 MPa, some conformational changes and aggregation appeared to occur, the rate of change being faster between 210 and 460 MPa. This resistance of the immunoglobulins may have pharmaceutical−medical applications. Only a small amount of aggregation of α-lactalbumin was observed below 500 MPa. This resistance to aggregation could be due to the absence of free SH groups and the initial difficulty in forming covalent links to other proteins.

Although few studies have been published on the inactivation of microorganisms in milk by pressure treatment (Cheftel, 1995), the available evidence indicates that treatments at 400−500 MPa are suitable for reducing the growth of microorganisms. On comparing pressure treatments in the present study with thermal treatment of goat's milk (Law, 1995; Montilla *et al.*, 1995), it can be seen that pressures of 400−500 MPa caused only a small amount of denaturation of immunoglobulins, serum albumin/lactoferrin, and α-lactalbumin, similar to that obtained by very mild heat treatment. However, pressures between 400 and 500 MPa caused high levels of denaturation of β-lactoglobulin that were only obtained after a fairly severe heat treatment of 80 °C for 10 min. β-Lactoglobulin, therefore, appeared particularly susceptible to high-pressure treatment and, as it is the main constituent of whey protein, almost 50% of total whey protein became irreversibly aggregated above 400 MPa (Figure 8).

In cow's milk, the activity of alkaline phosphatase is a useful index of the safety of heat treatment, as its thermal inactivation occurs after that of vegetative forms of pathogenic microorganisms. In goat's milk, however, the thermal inactivation of this enzyme is not suitable as an index of thermal treatment as it is inactivated at a lower temperature (Williams, 1986). In the present study, following pasteurization of goat's milk (72 °C for 15 s), alkaline phosphatase was completely inactivated whereas in raw milk or after pressure treatment (500 MPa for 10 min) the activity was unchanged (data not shown). Wong and Armstrong (1992) actually observed increased activity of microbial alkaline phosphatase at pressures up to 720 MPa.

DISCUSSION

Thermal and pressure treatments of milk could be distinguished by the different susceptibilities of the whey proteins to denaturation under the two treatments. On heat-treatment in this and previous studies (Law, 1995), the order of ease of irreversible denaturation of caprine whey proteins, based on loss of solubility at pH 4.6, was immunoglobulins > serum albumin/lactoferrin > β-lactoglobulin > α-lactalbumin. On pressure treatments, however, β-lactoglobulin was more readily denatured than the other whey proteins. At a pressure of 250 MPa at 25 or 50 °C for 10 min, β-lactoglobulin was extensively denatured whereas the other whey proteins showed little change in their solubility at pH 4.6. At a pressure of 500 MPa for 10 min at 25 °C, and especially at 50 °C, there were also appreciable increases in the levels of denaturation of the immunoglobulins and α-lactalbumin.

A further difference between thermal and pressure treatment was the appearance in the acid whey, after pressure treatment, of small soluble aggregates of β-lactoglobulin. On heat-treatment, denatured β-lactoglobulin becomes associated with κ-casein on the micelles and precipitates on subsequent acidification to pH 4.6. However, on pressure treatment where only part of the β-lactoglobulin was denatured, it also showed some tendency to form homopolymers or possibly polymers with other whey proteins. In studies of isolated β-lactoglobulin, Funterberger *et al.* (1995) similarly

found that pressure treatment resulted in formation of disulfide-linked polymers, and these contained between two and six subunits. In the present study, it was found that at higher pressures the amount of small polymers was reduced, possibly because of the formation of larger polymers that more readily precipitated at pH 4.6.

Previous studies on thermal treatment of milk have shown that there is a close correlation between the loss of solubility of denatured whey proteins at pH 4.6 and the extent to which they are incorporated into the rennet curd during cheesemaking. If pressure-denatured whey proteins were similarly incorporated, substantial increases in the yield of the formed curd and of the final ripened cheese could be obtained. The effects of the duration and temperature of the pressure treatment on whey protein denaturation, and the implications for cheesemaking, are being studied and will be reported in a future paper.

The activity of alkaline phosphatase in goat's milk is reduced by heat treatment but not by pressure treatment up to 500 MPa for 10 min at 25 °C or 50 °C. The differences in behavior of alkaline phosphatase and of the whey proteins on thermal and pressure treatments, therefore, may provide the basis of a test to determine the type and severity of treatments that have been applied to reduce the growth of microorganisms in a particular milk product.

ACKNOWLEDGMENT

We thank Foss-Electric Spain S. A. for lending us the Fluorophos-R Unit.

LITERATURE CITED

Cheftel, J. C. Review: High-pressure, microbial inactivation and food preservation. *Food Sci. Technol. Int.* **1995**, *1*, 75−90.

Desobry-Banon, S.; Richard, F.; Hardy, J. Study of acid and rennet coagulation of high pressurized milk. *J. Dairy Sci.* **1994**, *77*, 3267−3274.

Funtenberger, S.; Dumay, E.; Cheftel, J. C. Pressure-induced aggregation of β-Lactoglobulin in pH 7.0 buffers. *Lebensm.-Wiss. u.-Technol.* **1995**, *28*, 410−418.

Gay, M. F.; Jaubert, G.; Saboureau, S. Effect of pasteurization and microfiltration on the hygienic quality of goat's milk and on soft goat's cheeses. *Lait* **1993**, *73* (5−6), 499−509.

Hayakawa, I.; Kajihara, J.; Morikawa, K.; Oda, M.; Fujio, Y. Denaturation of bovine serum albumin (BSA) and ovalbumin by high pressure, heat and chemicals. *J. Food Sci.* **1992**, *57* (2), 288−292.

Hite, B. H. The effect of pressure in the preservation of milk. 1899, Bull. 58, 15−35. West Virginia Univ. Agr. Expt. Sta., Morgantown.

Howlett, J. R.; Ismail, A. A.; Armstrong, D. W.; Wong, P. T. T. Pressure-induced conformational changes in an antigen and an antibody and the implications on their use for hyperbaric immunoadsorption. *Biochim. Biophys. Acta* **1992**, *1159*, 227−236.

International Dairy Federation. Determination of alkaline phosphatase, 1992; Method 155.

Johnston, D. E.; Austin, B. A.; Murphy, R. J. Effects of high hydrostatic pressure on milk. *Milchwissenschaft* **1992**, *47* (12), 760−763.

Johnston, D. E.; Austin, B. A.; Murphy, R. J. Properties of acid-set gels prepared from high pressure treated skim milk. *Milchwissenschaft* **1993**, *48* (4), 206−209.

Law, A. J. R. Heat denaturation of bovine, caprine and ovine whey proteins. *Milchwissenschaft* **1995**, *50* (7), 384−388.

Law, A. J. R.; Brown, J. R. Compositional changes in caprine whey proteins. *Milchwissenschaft* **1994**, *49* (12), 674−678.

Law, A. J. R.; Leaver, J.; Banks, J. M.; Horne, D. S. Quantitative fractionation of whey proteins by gel permeation FPLC. *Milchwissenschaft* **1993**, *48* (12), 663−666.

Mertens, B. Developments in High Pressure Food Processing (part 2). *Int. Food Manuf. (ZFL)* **1993**, *44* (4), 182−187.

Montilla, A.; Balcones, E.; Olano, A.; Calvo, M. Influence of heat treatments on whey protein denaturation and rennet clotting properties of cow's and goat's milk. *J. Agric. Food Chem.* **1995**, *43*, 1908−1911.

Rowland, S. J. The heat denaturation of albumin and globulin in milk. II. Denaturation and degradation of protein at temperatures of 75−120 °C. *J. Dairy Res.* **1937a**, *8*, 1−5.

Rowland, S. J. The soluble protein fraction of milk. *J. Dairy Res.* **1937b**, *8*, 6−14.

Williams, D. J. A modification to the Aschaffenburg and Mullen alkaline phosphatase test suitable for goat's milk. *Aust. J. Dairy Tech.* **1986**, March, 28−30.

Wong, P. T. T.; Arsmtrong, D. W. FTIR spectroscopy kinetics analysis of alkaline phosphatase under hyperbaric manipulation. *Biochim. Biophys. Acta* **1992**, *1159*, 237−242.

Received for review June 6, 1996. Revised manuscript received December 9, 1996. Accepted December 19, 1996.[⊗] This study was funded in part by Research Contract AIR 1-CT92-0296 from the European Communities and by the Scottish Office Agriculture, Environment and Fisheries Department (SOAEFD).

JF960406O

[⊗] Abstract published in *Advance ACS Abstracts*, February 15, 1997.