Plasmin Activity in Pressurized Milk

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ABSTRACT

The effects of pressure (up to 400 MPa), applied at room temperature, on native proteinase activity of milk were investigated by means of plasmin activity, plasmin-derived activity after plasminogen activation and their distribution in different milk fractions, micelle microstructure, β -LG denaturation, and casein susceptibility to proteolytic attack. The pressure conditions assayed did not lead to plasmin inactivation and only decreased around 20 to 30% total plasmin activity after plasminogen activation. However, pressure caused severe disruption of the micellar structure, releasing high levels of caseins, plasmin, and plasminogen to the soluble fraction of milk. High levels of soluble denatured β -LG were also found in the ultracentrifugation supernatants of pressurized milks, particularly in those treated at 400 MPa. Probably as a result of micellar disintegration, caseins became more susceptible to proteolysis by exogenous plasmin. However, no enhanced proteolytic degradation was observed when we compared the evolution of pressurized and unpressurized milks during refrigerated storage. Serum-liberated plasmin may become more vulnerable to the action of proteinase inhibitors leading to a reduced proteolysis on refrigerated storage, despite the increased susceptibility of caseins to proteinase action.

(Key words: milk, plasmin system, micelle microstructure, β -LG denaturation)

Abbreviation key: CE = capillary electrophoresis, ϵ -ACA = ϵ -aminocaproic acid.

INTRODUCTION

The main native milk proteinase, plasmin, plays a very important role in the functional properties of dairy products, as it affects cheese yield, proteolysis during cheese ripening, yogurt quality, and keeping quality of UHT milk on storage (Bastian and Brown, 1996).

Plasmin, as well as its zymogen, plasminogen, are highly heat resistant, but plasmin activity in milk is driven by a complex system of activators and inhibitors with different heat stabilities. Treatment of milk at 72°C for 15 s inactivates plasmin by about 10 to 17%; however, it has been demonstrated that certain pasteurization conditions enhance proteolysis during subsequent storage, which was attributed to the thermal degradation of inhibitors operating in the plasmin system (Grufferty and Fox, 1988). In UHT milk, a heat treatment of 142°C for 18 s is necessary to completely prevent proteolysis by plasmin during storage of the product (Grufferty and Fox, 1988).

High pressure is gaining increasing acceptance as a food-processing technique because it exerts antimicrobial effects and causes reversible and irreversible changes in enzymatic activities, but the sensory and nutritional quality of food is not affected (Huppertz et al., 2002). In milk, plasmin activity was shown to resist at least 400 MPa applied for 30 min at 25°C (López-Fandiño et al., 1996), while these conditions reduced the total plasmin activity after plasminogen activation by 25% (García-Risco et al., 1998). Pressurization at higher temperatures considerably increased plasmin inactivation in milk, which reached 86.5% after treatments at 60°C (García-Risco et al., 2000). Plasmin was also barostable in buffer at room temperature, resisting up to 600 MPa for 20 min, but it was significantly inactivated at 400 MPa in the presence of β -LG (Scollard et al., 2000a).

Native proteinase activity in pressurized milk could also be driven by factors other than plasmin and plasminogen barostabilities. Pressurization at room temperature causes micellar disintegration and produces casein solubilization (López-Fandiño et al., 1998; Needs et al., 2000), although pressure treatments at temperatures over 40°C progressively increase micelle dimensions (García-Risco et al., 2000). When milk is pressure treated at room temperature, micelle disruption might enhance the susceptibility of casein to proteolysis by increasing the protein surface area available to the enzyme and the exposure of new substrate sites. Furthermore, these changes could also dissociate plasmin and plasminogen, normally attached to the casein micelles (Grufferty and Fox, 1988), to the serum. On the other

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hand, treatment of milk at pressures higher than 100 MPa induces β -LG denaturation, which increases with the temperature of the treatment (López-Fandiño et al., 1996; López-Fandiño and Olano, 1998), and unfolded β -LG can be a potent inhibitor of plasmin through thiol-disulfide bonding (García-Risco et al., 1998; Scollard et al., 2000a; Grufferty and Fox, 1988).

The objective of this work was to estimate the effects of pressure, applied at room temperature, on native proteolytic activity in milk. For this purpose, we have examined plasmin activity as well as plasmin-derived activity after plasminogen activation and their distribution in different milk fractions, micelle structure, β -LG denaturation, and casein susceptibility to proteolytic attack in pressurized and unpressurized milk.

MATERIALS AND METHODS

High-Pressure Treatments

Raw milk, collected from a local dairy farm, was skimmed by heating at 30°C for 30 min, centrifuged at $4500 \times g$ for 20 min at 20°C, and filtered through glass-wool. Milk was poured into 70-ml polyethylene bottles, avoiding any headspace, and pressurized at 100 to 400 MPa for 15 min (900 HP Eurotherm Automation, 69572 Dardilly Cedex, Lyon, France). The pressure was raised and released at a rate of 2.5 MPa/s. The temperature of the high-pressure treatment was $23 \pm 2^\circ\text{C}$, and it was controlled by circulating water through a jacket surrounding the pressure vessel. The experiments were repeated three times with milk from different batches, and every analytical determination was performed at least in triplicate.

Electron Microscopy

Unpressurized and pressurized liquid milk samples (5 ml) were mixed with 5 ml of 3% (wt/vol) agar solution. The mixture was poured on a petri dish, allowed to solidify, and cut into strips of $1 \times 10 \times 10 \text{ mm}^3$, approximately. The strips were fixed for 3 h in 2.5% (vol/vol) glutaraldehyde in PBS (pH 7.0) at 4°C, rinsed three times with PBS, and kept refrigerated overnight in PBS. Then the strips were postfixed in 1% (wt/vol) osmium tetroxide (Sigma Chemical Co., St. Louis, MO) for 1 h, rinsed three times with bidistilled water, and dehydrated with increasing concentrations of acetone (40, 60, 70, 90, 95, and 100%). Finally, they were infiltrated four times with a solution containing acetone and Spurr's epoxy resin (Sigma Chemical Co.) in proportions ranging from 3:1 acetone:resin to 100% resin, hardened for 72 h at 65°C, and cut into thin sections that were poststained with 2% uranyl acetate and Reynolds lead citrate. These were examined with a transmission elec-

tron microscope (Zeiss 902, Carl Zeiss, Oberkochen, Germany) operated at 80 kV. Micellar sizes were measured manually in $12 \times 15 \text{ cm}$ sections of the photographs taken at $50,000 \times$ magnification.

Protein Distribution Between Different Milk Fractions

Unpressurized and pressurized milk samples were ultracentrifuged at $100,000 \times g$ for 1 h at 4°C in a Beckman L-70 preparative ultracentrifuge (Beckman Instruments Inc., San Ramon, CA) using a type 70 Ti rotor. The supernatants were carefully removed and filtered through paper Whatman 41. Aliquots of the ultracentrifugation supernatants were treated with 2 M HCl to pH 4.6, followed by centrifugation at $4500 \times g$ for 15 min and filtration through Whatman 41. Denaturation of β -LG was calculated from the reduction in peak area following pH adjustment to 4.6.

Capillary electrophoresis (CE) was performed as described by Recio and Olieman (1996) with a Beckman P/ACE System MDQ (Beckman Instruments Inc., Fullerton, CA). Separations were carried out at pH 3.0 with a hydrophilic-coated fused-silica capillary (CElect P1, Supelco, Bellefonte, PA) of $0.60 \text{ m} \times 50 \mu\text{m}$, with a slit opening of $100 \times 800 \mu\text{m}$, (0.50 m to detection point), at a temperature of 45°C, with a linear voltage gradient of 0 to 25 kV in 3 min, followed by constant voltage of 25 kV. Protein identification was carried out according to Recio et al. (1997).

Plasmin Activity

The distribution of plasmin and plasmin-derived activity after plasminogen activation by urokinase in the serum and casein fractions of pressurized and unpressurized milks was examined by combining the conventional and modified methodologies proposed by Politis et al. (1993). Skim milk was divided in two aliquots. The first aliquot was incubated with 50 mM ϵ -aminocaproic acid (ϵ -ACA) (Sigma Chemical Co.) for 2 h at room temperature to dissociate plasmin and plasminogen from casein micelles and allow their transfer into the serum fraction. The second aliquot was not treated with ϵ -ACA, so that release of plasmin and plasminogen activities to the serum was not induced. After incubation, the milk samples were ultracentrifuged at $100,000 \times g$ for 1 h at 4°C, and the supernatants were carefully removed and filtered as described above.

The casein pellets were reconstituted to the original volume (35 ml) in 50 mM Tris buffer (pH 8.0) containing 110 mM NaCl and were incubated at room temperature with 50 mM ϵ -ACA to allow the transfer to the buffer of plasmin and plasminogen remaining in the casein

micelles. The casein suspensions were then ultracentrifuged at $100,000 \times g$ for 1 h at 4°C, and the filtered supernatants were used for the determination of the enzymatic activities.

Plasmin activity and plasmin-derived activity after plasminogen activation by urokinase were determined by the method originally developed by Rollemma et al. (1983) and modified by Korycka-Dahl et al. (1983). Milk serum or buffer (50 μ l) was mixed with 950 μ l of 50 mM Tris buffer (pH 7.4) containing 110 mM NaCl, 2.5 mM ϵ -ACA and 0.6 mM H-D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride (V-0882, Sigma Chemical Co.). For the determination of plasmin-derived activity, 150 plough units of urokinase (U-5004, Sigma Chemical Co.) were also added to achieve plasminogen activation. The reaction mixtures were incubated at 37°C, and the absorbances at 405 nm were measured during 3 h in an ELISA plate reader (Multiskan Ascent, Labsystems, Barcelona, Spain). One unit of activity was defined as the amount of enzyme that produces a change in absorbance of 0.001 in 1 min under the assay conditions.

Casein Susceptibility to Plasmin Action

Susceptibility to proteolysis with plasmin was conducted by incubating 10 ml of unpressurized and pressurized skim milks with 75 μ l of plasmin from bovine plasma (5 I.U./ml, P-7911, Sigma Chemical Co.) at 37°C during 90 min. Aliquots (1 ml) were withdrawn from the mixtures at intervals (0, 10, 30, 60, and 90 min), and the reactions were stopped by addition of the sample buffer (dilution 1:1.5) used for the CE analysis (Recio and Olieman, 1996).

Changes underwent by unpressurized and pressurized milks on storage, as a result of the action of native plasmin, were compared by keeping milk at refrigeration temperatures (5°C) in the presence of 30 mM NaN₃ as inhibitor of the bacterial growth. Samples were taken for analysis at 2-d intervals for 6 d.

Proteolysis of main caseins and appearance of degradation products was determined by CE as explained above. For the purpose of normalization, results were expressed as the quotients of the peak areas of the main degradation products: the sum of γ_2 -casein + γ_3 -casein and α_{SP} -casein divided by the area of α -lactalbumin, because the area of this protein did not change as a result of the pressure or enzymatic treatments.

RESULTS

Pressure-Induced Changes in Micelles and Whey Proteins

Figure 1 shows transmission electron micrographs of casein micelles in unpressurized skim milk and milks

treated at 100, 200, 300, and 400 MPa for 15 min. As a result of pressure, average micelle diameters were reduced from approximately 150 to 300 nm in unpressurized milk to 75 to 230 nm in milk pressurized at 100 MPa, 100 nm at 200 MPa, and 40 nm at 300 and 400 MPa. In addition to decreasing micelle dimensions, pressurization led to a narrower size distribution. Thus, the presence in milk treated at 100 MPa of micelles with a size similar to that of the unpressurized milk suggested that only some of the micelles were fragmented, while the high size uniformity found in milk subjected to 300 and 400 MPa, indicated that most of the micelles were disrupted at those pressures. Furthermore, clusters of micelles were found in milk pressurized at 300 MPa, which were dispersed at 400 MPa. A decrease in the average diameter of particles after high-pressure treatment of milk samples at room temperature was already reported, with maximum disruption also occurring at 300 MPa (García-Risco et al., 2000; Needs et al., 2000; Scollard et al., 2000b). Pressure-induced micellar disruption is attributed either to breakage of linkages between casein and inorganic constituents and/or hydrophobic bonds (Schrader et al., 1997).

As a result of micelle disintegration induced by high pressure, an important release of casein to the soluble fraction was produced as shown by CE of the ultracentrifugation supernatants of unpressurized and pressurized milks (Figure 2). The concentration of individual caseins in the ultracentrifugation sera of milks pressurized at 200 and 400 MPa was very similar, despite the differences found in micellar size. This is in agreement with previous results that also showed that maximum levels of nonsedimentable caseins, Ca, P, and Mg, are found in the serum of milk treated at 300 MPa (López-Fandiño et al., 1998). The individual casein contents were found in the order β -casein > κ -casein > α_{S1} -casein > α_{S0} -casein > α_{S2} -casein.

In addition to nonsedimentable caseins, 98 and 80% of β -LG remained soluble in ultracentrifugation sera of milks treated at 200 and 400 MPa (Figure 2). Adjustment of the pH of the ultracentrifugation supernatants to 4.6 revealed that 12 and 78% of soluble β -LG was denatured at 200 and 400 MPa, respectively.

Plasmin Activity

Plasmin activity and plasmin-derived activity after plasminogen activation were determined in the serum and casein fractions of milk samples incubated in the absence and presence of ϵ -ACA (Table 1). In unpressurized milk samples, approximately 85% of plasmin activity and 82% of the total plasmin activity produced after plasminogen activation were associated with the casein

Figure 1. Transmission electron micrographs of unpressurized milk (a) and milk pressurized at 100 (b), 200 (c), 300 (d), and 400 MPa (e). The bar corresponds to 0.2 μm ; M: casein micelle.

Figure 2. Electropherograms of the ultrafiltration supernatants of unpressurized (a) and pressurized milk at 200 (b), and 400 MPa (c). 1, α -Lactalbumin; 2, β -Lactoglobulin; 3, α_{S2} -casein; 4, α_{S1} -casein; 5, α_{S0} -casein; 6, κ -casein; 7, β -casein A¹; 8, β -casein A².

Figure 3. Main degradation products formed during the incubation with exogenous plasmin of unpressurized (black triangle) and pressurized milk samples at 200 (black square) and 400 (black circle) MPa. Results are expressed as normalized peak areas in the capillary electrophoresis electropherograms of γ_2 -casein + γ_3 -casein/ α -lactalbumin (a) and α_{SP} -casein/ α -lactalbumin (b), with vertical bars (sometimes overlapped by the symbols) showing the standard deviation.

Table 1. Plasmin and plasmin-derived activity after plasminogen activation in the serum and reconstituted casein fractions of unpressurized and pressurized milk samples incubated in the absence and presence of ϵ -aminocaproic acid (ϵ -ACA). Results are means followed by standard deviations into brackets.

Plasmin activity	Milk incubated without ϵ -ACA		Milk with ϵ -ACA	
	Serum	Casein	Serum	Casein
Unpressurized	1.88 (0.40)	9.96 (1.00)	5.64 (0.74)	3.30 (0.50)
Pressurized 200 MPa	4.54 (0.56)	5.28 (0.48)	6.44 (1.58)	2.76 (0.82)
Pressurized 400 MPa	6.86 (0.82)	5.12 (1.44)	7.12 (1.26)	2.18 (0.62)
Plasmin-derived activity after plasminogen activation				
Unpressurized	11.5 (0.54)	53.32 (2.38)	44.28 (2.70)	14.90 (2.38)
Pressurized 200 MPa	25.16 (1.32)	25.76 (2.70)	40.36 (0.94)	9.04 (0.72)
Pressurized 400 MPa	28.98 (3.06)	21.66 (2.30)	34.52 (2.34)	7.82 (0.96)

micelles, as revealed by the measurements performed in the absence of ϵ -ACA. The addition of ϵ -ACA caused approximately 65 to 75% of plasmin activity and of the total plasmin activity obtained after plasminogen activation to be transferred to milk serum. The sum of activities in the serum and casein fractions was lower when the milk samples were initially incubated with ϵ -ACA. This is probably because, in that case, most of the activity was estimated after it was released to the serum, where there are specific inhibitors of the plasmin system (Politis et al., 1993; Precetti et al., 1997).

The measurement of plasmin activity and plasmin-derived activity after plasminogen activation in the serum phases of milks treated at 200 and 400 MPa that were not incubated with ϵ -ACA revealed a pressure-induced release of micellar bound enzymes to the serum fractions. Other processes that cause micellar disruption, such as the addition of acids or the growth of psychrotrophic bacteria, also bring about a shift in plasmin from the casein to the whey fraction, which can affect the quality of milk and its derived products (Fajardo-Lira et al., 2000; Hayes and Nielsen, 2000). In samples incubated with ϵ -ACA, plasmin activities measured in the serum and in the casein fractions of milks pressurized at 200 and 400 MPa were closer to that of the unpressurized milk. This indicates that overall plasmin activity did not change considerably as a result of the pressures applied but underwent a different distribution. In fact, it has been reported that plasmin resists pressures of 400 MPa in milk (García-Risco et al., 2000; Scollard et al., 2000b). Nevertheless, the total plasmin activity after plasminogen activation decreased by 20 to 30% with pressurization, which is also in agreement with previous results (García-Risco et al., 1998).

Casein Susceptibility to Plasmin Action

Susceptibility of casein to proteolytic attack was estimated by CE after incubation with exogenous plasmin

for up to 90 min at 37°C. As expected, the main degradation products were γ_2 - and γ_3 -caseins, that result from the action of the enzyme of β -caseins, as well as another fragment, named α_{SP} , that arises from proteolysis of α_{S1} -casein (Recio et al., 1997). No proteolytic degradation was detected in unpressurized and pressurized milk samples incubated without exogenous plasmin during the same period. Figure 3 illustrates the evolution of those breakdown products during incubation with the enzyme. One-way ANOVA of the data showed that pressurization at 200 and 400 MPa made caseins significantly ($P < 0.05\%$) more vulnerable to the proteolytic attack. Proteolysis was enhanced in milk treated at 400 MPa over milk pressurized at 200 MPa ($P < 0.05\%$).

A slight proteolytic degradation took place in the milk samples during 6 d of refrigerated storage due to the action of native plasmin, but we did not find significant quantitative differences in the rates of protein breakdown between unpressurized and pressurized milks (results not shown), which is in agreement with previous works (García-Risco et al., 1998; García-Risco et al., 2000). Scollard et al. (2000b) reported that at pressures of 300 to 400 MPa proteolysis was increased relative to raw milk, which also suggested an enhanced availability of substrate bonds to plasmin. However, in that case the statistical signification of the differences observed was not reported.

DISCUSSION

Our results suggest that pressurization made caseins more susceptible to the proteolytic attack. This is not surprising because pressure caused severe disruption of the micellar structure, releasing high levels of soluble caseins. This can make the access of proteinases to caseins easier and so increase the degree of proteolysis. However, no enhanced proteolytic degradation was observed, when we compared the evolution of pressurized and unpressurized milks during refrigerated storage.

It should also be noted that, as a consequence of these deep modifications in the micellar structure, not only individual caseins, but also plasmin and plasminogen were released in the soluble fraction of milk on pressurization. Even if the pressure conditions assayed did not lead to plasmin inactivation and only decreased by 20 to 30% of the total plasmin activity after plasminogen activation, it is likely that serum-liberated enzymes became more vulnerable to the action of proteinase inhibitors normally found in the soluble fraction. This could lead to a diminished proteinase activity that could counteract the enhanced susceptibility of caseins to the enzyme, resulting in proteolysis levels on refrigerated storage similar to those undergone by unpressurized milk.

High levels of soluble denatured β -LG were found in the ultracentrifugation supernatants of pressurized milks, particularly in those treated at 400 MPa. Whether the denatured β -LG found in the soluble fraction is attached to the pressured-dissociated caseins or polymerized between itself remains unclear. Scollard et al. (2000b), using transmission electron microscopy coupled with immunogold labeling of β -LG, found β -LG dots close to disrupted micelle fragments, but definitive evidence for association is lacking. It has been suggested that in β -LG containing systems pressure inactivation of plasmin may be due to thiol-disulphide bonding with unfolded β -LG (García-Risco et al., 1998; Scollard et al., 2000a) in a way similar to what occurs when it is exposed to heat (Grufferty and Fox, 1988). Nevertheless, plasmin inactivation does not parallel β -LG denaturation, the latter being considerably faster (López-Fandiño et al., 1996; Scollard et al., 2000b). Furthermore, plasmin is more pressure resistant in milk than in buffers incorporating β -LG, which has been attributed to a protective effect due to pressure-induced self-aggregation of β -LG and/or an increase in the available interaction sites of β -LG with disrupted casein (Scollard et al., 2000b).

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REFERENCES

- Bastian, E. D., and R. J. Brown. 1996. Plasmin in milk and dairy products: An update. *Int. Dairy J.* 6:435–457.
- Fajardo-Lira, C., M. Oria, K. D. Hayes, and S. S. Nielsen. 2000. Effect of psychrotrophic bacteria and of an isolated protease from *Pseudomonas fluorescens* M3/6 on the plasmin system of fresh milk. *J. Dairy Sci.* 83:2190–2199.
- García-Risco, M. R., E. Cortés, A. V. Carrascosa, and R. López-Fandiño. 1998. Microbiological and chemical changes in high-pressure-treated milk during refrigerated storage. *J. Food Prot.* 61:735–737.
- García-Risco, M. R., A. Olano, M. Ramos, and R. López-Fandiño. 2000. Micellar changes induced by high pressure. Influence in the proteolytic activity and organoleptic properties of milk. *J. Dairy Sci.* 83:2184–2189.
- Grufferty, M. B., and P. F. Fox. 1988. Milk alkaline proteinase. *J. Dairy Res.* 55:609–630.
- Hayes, K. D., and S. S. Nielsen. 2000. Plasmin levels in fresh milk whey and commercial whey protein products. *J. Dairy Sci.* 83:387–394.
- Huppertz, T., A. L. Kelly, and P. F. Fox. 2002. Effects of high pressure on constituents and properties of milk. *Int. Dairy J.* 12:561–572.
- Korycka-Dahl, M., B. Ribadeau-Dumas, N. Chene, and J. Martal. 1983. Plasmin activity in milk. *J. Dairy Sci.* 66:704–711.
- López-Fandiño, R., A. V. Carrascosa, and A. Olano. 1996. The effects of high pressure on whey protein denaturation and the cheese-making properties of raw milk. *J. Dairy Sci.* 79:929–936.
- López-Fandiño, R., M. A. de la Fuente, M. Ramos, and A. Olano. 1998. Distribution of minerals and proteins between the soluble and colloidal phases of pressurized milks from different species. *J. Dairy Res.* 65:69–78.
- López-Fandiño, R., and A. Olano. 1998. Effects of high pressures combined with moderate temperatures on the rennet coagulation properties of milk. *Int. Dairy J.* 8:623–627.
- Needs, E. C., R. A. Stenning, A. L. Gill, V. Ferragut, and G. T. Rich. 2000. High pressure treatment of milk: effects on casein micelle structure and on enzymic coagulation. *J. Dairy Res.* 67:31–42.
- Politis, I., B. Zavizion, D. M. Barbano, and R. C. Gorewit. 1993. Enzymatic assay for the combined determination of plasmin plus plasminogen in milk: revisited. *J. Dairy Sci.* 76:1260–1267.
- Precetti, A. S., M. P. Oria, and S. S. Nielsen. 1997. Presence in bovine milk of two protease inhibitors of the plasmin system. *J. Dairy Sci.* 80:1490–1496.
- Recio, I., L. Amigo, M. Ramos, and R. López-Fandiño. 1997. Application of capillary electrophoresis to the study of proteolysis of caseins. *J. Dairy Res.* 64:221–230.
- Recio, I., and C. Olieman. 1996. Determination of denatured serum proteins in the casein fraction of heat-treated milk by capillary zone electrophoresis. *Electrophoresis* 17:1228–1233.
- Rollema, H. S., S. Visser, and J. K. Poll. 1983. Spectrophotometric assay of plasmin and plasminogen in bovine milk. *Milchwissenschaft* 38:214–217.
- Scollard, P. G., T. P. Beresford, P. M. Murphy, and A. L. Kelly. 2000a. Barostability of milk plasmin activity. *Lait* 80:609–619.
- Scollard, P. G., T. P. Beresford, E. C. Needs, P. M. Murphy, and A. L. Kelly. 2000b. Plasmin activity, β -lactoglobulin denaturation and proteolysis in high pressure treated milk. *Int. Dairy J.* 10:835–841.
- Schrader, K., W. Buchheim, and C. V. Morr. 1997. High pressure effects on the colloidal calcium phosphate and the structural integrity of micellar casein in milk. Part 1. High pressure dissolution of colloidal calcium phosphate in heated milk systems. *Nahrung* 41:133–138.