ELSEVIER

PII: S0308-8146(96)00303-2

Food Chemistry, Vol. 59, No. 3, pp. 439–447, 1997

0308-8146/97 $17.00 + 0.00

Combined effects of temperature and high-pressure treatments on physicochemical characteristics of skim milk

F. Gaucheron,[a]* M. H. Famelart,[a] F. Mariette,[b] K. Raulot,[a] F. Michel[a] & Y. Le Graet[a]

[a]*INRA, LRTL, 65 rue de Saint Brieuc, 35042 Rennes, France*
[b]*CEMAGREF, Division Technologie, 17 rue de Cucillé, 35044 Rennes, France*

(Received 26 April 1996; revised version received 5 September 1996; accepted 5 September 1996)

The combined effects of temperature (4, 20 and 40°C) and high-pressure treatments (250, 450 and 600 MPa for 30 min) on the physicochemical characteristics of skim milk were studied. At 4, 20 and 40°C, the effects of high-pressure treatments were similar (except for the treatment at 250 MPa), leading to an increase in protein hydrophobicity, a decrease in lightness, a decrease in average diameter of particles and slight solubilization of calcium and phosphorus from the colloidal to the aqueous phase of the milk. At the same time, denaturation of β-lactoglobulin probably occurred. At 40°C, and especially at 250 MPa, the effects were very different because protein hydrophobicity remained unchanged and the average diameter of particles increased with the presence of two distinct populations of casein micelles. These results are discussed in relation to the effect of high-pressure treatments on protein structure.

INTRODUCTION

The effects of high-pressure treatment on protein stability are governed by Le Chatelier's principle, which indicates that a volume decrease with increasing pressure will tend to adjust itself to nullify that change. At the same time, reversible and irreversible conformational changes may occur by disruption or stabilization of hydrophobic interactions, ionic and hydrogen bonds (Carter *et al.*, 1978; Masson, 1992; Weber, 1992; Gross & Jaenicke, 1994). Many studies have been performed on food proteins using high-pressure treatment (Cheftel, 1992; Tausher, 1995), and several experiments on milk have been reported. Physicochemical analyses reveal that the application of high hydrostatic pressure at a temperature of 20°C to skim milk leads to a decrease in the mean hydrodynamic diameter of casein particles, a decrease in turbidity and lightness, and an increased viscosity (Johnston *et al.*, 1992; Desobry-Banon *et al.*, 1994). A parallel increase in exposure of hydrophobic groups occurs (Johnston *et al.*, 1992). These modifications are probably due to a conformational change of casein micelles (Shibauchi *et al.*, 1992; Buccheim & Prokopeck, 1992). Values for soluble calcium concentration after high-pressure treatment are contradictory. According to Johnston *et al.* (1992), there is a slight increase in calcium activity between 200 and 600 MPa, whereas, according to Desobry-Banon *et al.* (1994), the soluble calcium concentration is maximal (0.6 g litre^{-1}) at 250 MPa. Recently, Lee *et al.* (1996) showed that the colloidal structure of the calcium caseinate micelles is also drastically disrupted in a similar manner to that observed for the native casein micelles in skim milk.

The properties of acid-induced gels and of rennet gels prepared from high-pressure-treated skim milk have also been investigated. The acidification of pressurized milk by gluconic acid δ-lactone induces a faster coagulation than with unpressurized milk (Desobry-Banon *et al.*, 1994). The gel strength obtained increases with increasing high-pressure (Johnston *et al.*, 1993; Desobry-Banon *et al.*, 1994) and a resistance to syneresis was observed. High-pressure treatments (above 230 MPa) lead to a shorter rennet clotting time (Shibauchi *et al.*, 1992; Desobry-Banon *et al.*, 1994).

Temperature is also a physical variable which affects the physicochemical properties of milk (Walstra & Jenness, 1984). This study therefore attempts to examine the effects of high-pressure treatments combined with temperature on the physicochemical characteristics of skim milk. Complementary results obtained with whey protein-free milk are also described.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Milk and native phosphocaseinate suspension

Reconstituted milk was prepared from a low-heat skim milk powder at a concentration of 9.37% (w/w). The native phosphocaseinate powder, obtained from a milk retentate enriched in native casein micelles by microfiltration, was supplied by P. Schuck (Schuck *et al.*, 1994). The native phosphocaseinate suspension was reconstituted in a milk ultrafiltrate to produce a casein concentration of 25 g litre^{-1}; 0.01% thimerosal (Sigma Chemical Co., St Louis, MO, USA) was added to prevent bacterial growth.

High-pressure treatment

The reconstituted milk or native phosphocaseinate suspension was packed into flexible plastic bottles without headspace. Pressure treatments were performed at 250, 450 and 600 MPa in a hydrostatic pressure unit (ACB; GEC Alsthom, Nantes, France). The samples were pressurized for 30 min. Just before high-pressure treatment, the milk samples were at 20°C. The temperature of the hydrostatic pressure unit was controlled at 4, 20 or 40°C. Previous experiments using a thermocouple placed inside the sample showed that a pressurization time of 15 min was required for the milk samples to reach temperatures of 4, 20 or 40°C. After high-pressure treatment, samples were stored overnight at 20°C before analysis. It is worth nothing that, between 5 h and 45 h after pressurization, the lightness of the samples was constant under these storage conditions. Pressurization of milk samples and subsequent analyses were carried out in triplicate.

Analyses

Spectrofluorimetric measurement

Exposure of hydrophobic groups of proteins was measured by the fluorescent probe binding method of Bonomi *et al.* (1988). Briefly, spectrofluorimetric measurements were taken on an LS 50B spectrofluorimeter (Perkin-Elmer, Quentin-en-Yvelines, France). The fluorophor 8-anilino-1-naphthalenesulphononic acid (ANS; Sigma) was used; when interacting with hydrophobic sites of proteins, its fluorescence parameters were an excitation wavelength of 390 nm and an emission wavelength of 480 nm. Aliquots of treated milk samples (1 ml) were diluted with 50 mM potassium phosphate buffer, pH 6.8, to a final volume of 10 ml, and titrated at room temperature with small aliquots of an aqueous solution of ANS (final concentration between 0 and 300 μM) until no further increase in fluorescence was observed. The emission and excitation slits were both set at 2.5 nm bandwidth. Analysis of the binding data allowed the maximum fluorescence reached at saturating ANS concentration (F_{max}, i.e. asymptotic value of the titration curve) to be determined. This value was taken as 100 for unpressurized milk samples.

Lightness measurement

A microcolour tristimulus colorimeter (Chromameter CR-300; Minolta, Carrières-sur-Seine, France) was used for colour testing. Calibration was performed using the Minolta calibration plate (standard tristimulus values: $Y = 92.4$; $x = 0.3161$; $y = 0.3325$). Results were expressed using the L^* value according to CIE-LAB (Commission Internationale de l'Éclairage, 1971). In this system, L^* defines the position of the sample on the light–dark axis.

Particle size analysis

A laser granulometer (N4MD Coulter; Coultronics, Margency, France) was used to evaluate the average diameters of particles at 20°C. The milk sample was diluted in 20 mM imidazole–HCl buffer pH 6.6, containing 5 mM $CaCl_2$ and 50 mM NaCl; after stabilization for 10 min inside the cell holder, light intensity scattering at 90° was measured for 600 s.

Observation of casein micelles by transmission electron microscopy was made on a Philips CM12 apparatus (Philips Industrie, Bobigny, France) in transmission mode, at 80 kV. Milk samples were diluted 1/250 in 0.2 M sodium cacodylate buffer at pH 7.4. An aliquot was dropped onto a collodion-treated carbon grid (300 mesh, 3 mm diameter). After 30 s, excess sample was removed on a filter paper and the grid was immersed in a 2% (w/v) uranyl acetate solution for 2 min. The grid was drained and then air-dried. Multiple fields were viewed and typical fields photographed.

Determination of hydration of ultracentrifuged pellet

Each milk sample (including unpressurized sample) was centrifuged at 77 000*g* (Beckman 50.2 Ti rotor) for 2 h in a Beckman L8 ultracentrifuge at 20°C. Supernatants were carefully separated from the pellet. Then, drained pellets were weighed and dried at 103°C for 7 h to remove water of hydration. The difference between the weight before and after drying, expressed as grams of water per gram of dry pellet was taken as the water of hydration.

Determination of calcium and phosphorus content

The calcium content in the ultracentrifugal supernatant (filtered through a 0.45 μm filter) was determined by atomic absorption spectrometry (Varian AA 300 equipment; Les Ulis, France) according to Brulé *et al.* (1974). The phosphorus content in the ultracentrifugal supernatants was determined according to the standard method of the International Dairy Federation (1987).

Casein analysis by reversed-phase high-performance liquid chromatography (RP-HPLC)

Caseins were separated by RP-HPLC on a C_4 column (Vydac 214 TP 5415; Touzart Matignon, Vitry-sur-Seine,

France), 150 mm×4.6 mm internal diameter, particle size 5 μm, at 40°C, using gradient elution with acetonitrile at a flow rate of 1 ml min^{-1}. Solution A was trifluoroacetic acid dissolved in double-distilled water (1 ml $litre^{-1}$); solution B was 1 ml of trifluoroacetic acid dissolved in acetonitrile–double-distilled water (80:20, v/v). Milk samples and ultracentrifugation supernatants were diluted 20-fold and 4-fold, respectively. After equilibration of the column with 37% of solution B, caseins (50 μl) were applied and eluted by increasing the concentration of solution B as follows: 0–30 min, 37–54%; 30–35 min, 54–100%. Eluted peaks were detected by absorbance at 214 nm. It is worth noting that RP-HPLC analyses of caseins are commonly performed after dissolution of the sample in a buffer containing a reducing agent (e.g. mercaptoethanol or dithiothreitol) to improve the chromatographic separation (Léonil *et al.*, 1995). In this case, as the aim was to visualize potential interactions between proteins via intermolecular disulphide bonds, this chemical reduction was not performed.

Nitrogen analysis

The non-casein nitrogen and non-protein nitrogen contents were determined as the nitrogeneous compounds that were soluble at pH 4.6 and in 12% trichloroacetic acid solution, respectively. These analyses were performed on milk, before and after high-pressure treatment, using the Kjeldahl method. The non-casein protein concentration was calculated by multiplying the non-casein nitrogen concentration by the factor 6.38.

α-Lactalbumin and β-lactoglobulin analysis by gel permeation HPLC (GP-HPLC)

α-Lactalbumin and β-lactoglobulin of the ultracentrifugation supernatant were separated and quantified by GP-HPLC using a Progel-TSK G3000 column (Supelco, Saint Germain-en-Laye, France). Samples were diluted 4-fold and eluted with 30% acetonitrile in water containing 0.1% trifluoroacetic acid. Eluted peaks were detected by absorbance at 214 nm.

RESULTS AND DISCUSSION

Global effects of high-pressure treatment in combination with temperature on milk

ANS is known to bind hydrophobic areas of proteins accessible to the aqueous solvent. Upon binding, its fluorescence is drastically enhanced so that exposed hydrophobic surface areas can be quantitatively determined. The exposure of hydrophobic regions of milk proteins measured by binding of ANS as a function of high-pressure treatment and of temperature is presented in Fig. 1. At 4°C, the F_{max} values increased with high-pressure treatment up to 450 MPa, then slightly decreased with treatment at 600 MPa. At 20°C, there was an increase in F_{max} values from 0 to 600 MPa. The increase was lower at 20°C than at 4°C. Surprisingly, at 40°C, the F_{max} value remained unchanged between 0 and 250 MPa; then it increased up to 600 MPa. These variations in F_{max} indicate structural modifications to the milk proteins because, when a protein is partially or completely disorganized, hydrophobic segments may be differently exposed. However, the modified proteins were not determined. It is worth nothing that Johnston *et al.* (1992), who studied the conformational changes of milk proteins induced by application of hydrostatic pressure up to 600 MPa at 20°C, have also reported an increase in F_{max} value.

Effects of high-pressure treatment in combination with temperature on casein micelles in milk or in native phosphocaseinate suspension

The lightness of the different milk samples as a function of high-pressure treatment and temperature is shown Fig. 2. Globally, lightness decreased up to a high-pressure treatment of 450 MPa. These decreases in lightness were lower at 40°C than at 20°C and 4°C. Then, between 450 and 600 MPa, the lightness was constant.

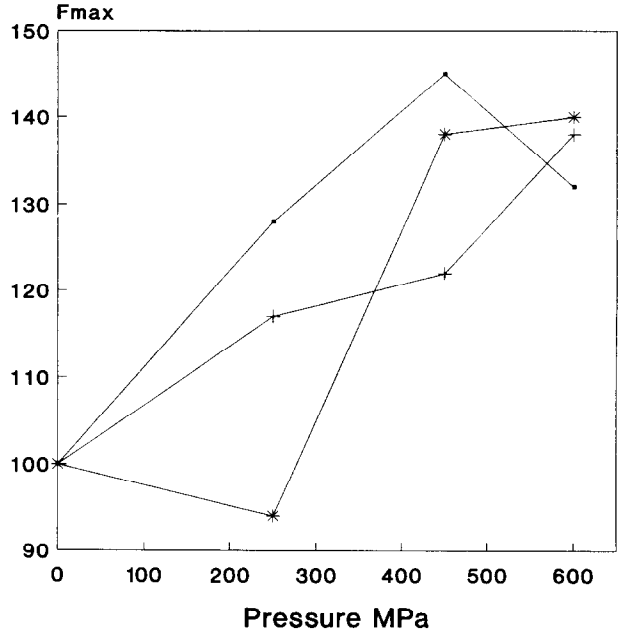

Fig. 1. Combined effects of temperature and high-pressure treatment on the exposure of hydrophobic regions of milk proteins. Hydrophobicity was measured by the binding of fluorescent probe 8-anilino-1-naphthalenesulphonate (ANS) to proteins according to the method described by Bonomi *et al.* (1988). Analysis of binding data allowed the determination of the maximum fluorescence, F_{max}, attainable at saturated ANS concentration (F_{max} corresponds to the asymptotic value of the titration curve). The F_{max} of unpressurized milk was taken as 100. The accuracy of the F_{max} determination was ±10. The temperature of the hydrostatic pressure unit was regulated at 4°C (■), 20°C (+) or 40°C (*). Samples were pressurized for 30 min and stored overnight at 20°C before analysis.

At 20°C, the decrease in lightness was similar to that observed by Johnston *et al.* (1992) and Desobry-Banon *et al.* (1994). It should be noted that high-pressure treatment of 250 MPa carried out at 4, 20 or 40°C on native phosphocaseinate suspension (corresponding to a whey protein-free milk) induced the same decreases in lightness as those observed with milk samples (results not shown). On the other hand, decreases in milk lightness were also observed when casein micelle disintegration was induced by calcium-chelating agents such as sodium salts of hexametaphosphate, orthophosphate, citrate and EDTA (Johnston & Murphy, 1992). Thus, from these decreases in lightness after high-pressure treatment, and Rayleigh's equation (which shows that light scattering is dependent on the 6th power of the particle radius), it can be deduced that a decrease in particle size occurred. Moreover, these structural changes in milk were irreversible, because the initial lightness was never recovered even after 45 h of storage at 20°C. Using spectrofluorimetric measurements in the presence of ANS, Johnston *et al.* (1992) showed that the effect of high-pressure treatment on the exposure of hydrophobic groups persisted for at least 8 days. A comparison with other proteins indicates that high-pressure treatment also induced structural changes in protein aggregates which can be reversible or irreversible: this was shown by the dissociation and inhibition of multimeric enzymes after high-pressure treatment (Penniston, 1971) or by the pressure-induced dissociation of brome mosaic virus (Silva & Weber, 1988). On the other hand, Silva *et al.* (1986) indicated that, after decompression, complete reassociation of tryptophan synthase β_2 subunits takes place. In the same way, Ruan and Weber (1989) described the total recovery of enzyme activity of yeast glyceraldehyde phosphate dehydrogenase after high-pressure treatment and decompression.

The combined effects of temperature and high pressure treatments on the average diameter of particles obtained by laser granulometry are shown in Fig. 3. High-pressure treatment at 250 MPa resulted in different particle sizes, depending on the temperature. At 4, 20 and 40°C, the average diameter of particles was 133, 184 and 252 nm, respectively. Under the same conditions of pressurization, changes in particle size were also observed by transmission electron microscopy (Fig. 4). For the unpressurized milk sample, the micelle size was about 100 nm (Fig. 4(A)). After high-pressure treatment of 250 MPa at 4°C and 20°C, particle sizes of about 50 nm were determined (Figs 4(B) and (C), respectively). After high-pressure treatment of 250 MPa at 40°C, two distinct populations were detected (Fig. 4(D)), with particle sizes of about 50 nm and 250 nm (arrows, Fig. 4(D)). These lower diameter values obtained with electron microscopy compared to the mean size of particles obtained with laser granulometry arise from sample dehydration (Pierre *et al.*, 1995). The presence of small particles would explain the decrease in the apparent lightness (Fig. 2), and the presence of large particles would explain the observed increase in the average diameter of particles determined by laser granulometry (Fig. 3). On the other hand, high-pressure treatment of

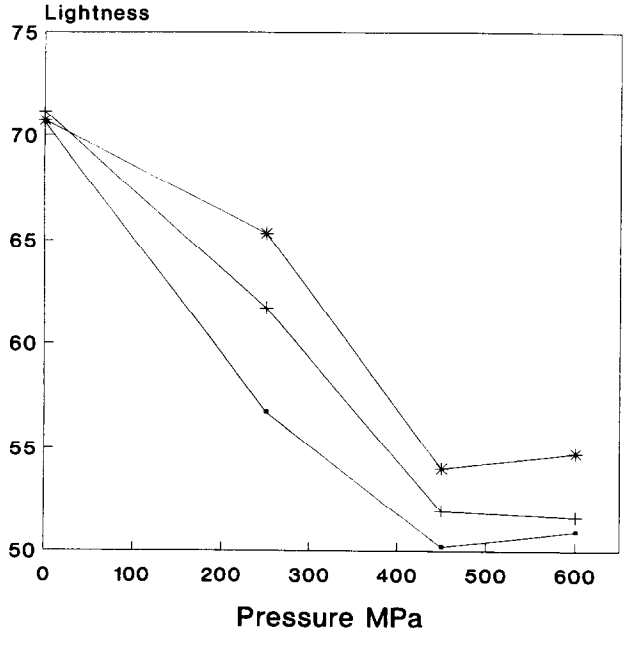

Fig. 2. Combined effects of temperature and high-pressure treatment on the milk lightness. The accuracy on the lightness value was ± 2 units. Complete conditions of high-pressure treatment and sample storage are described in the legend of Fig. 1.

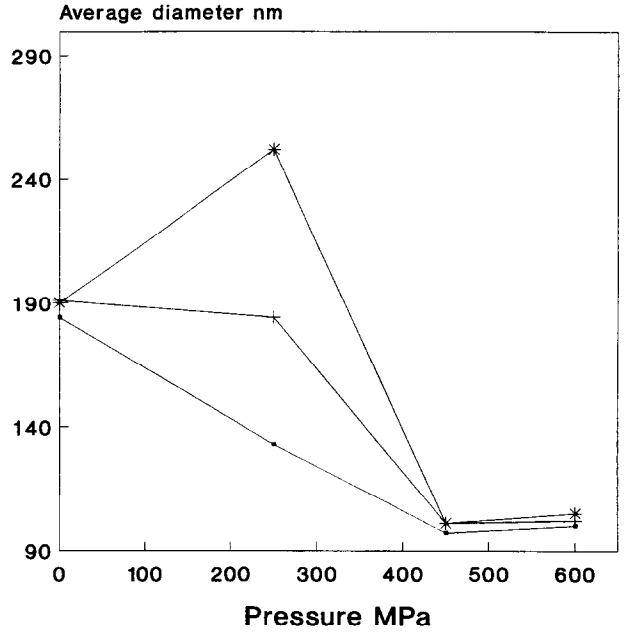

Fig. 3. Combined effects of temperature and high-pressure treatment on the average diameter of particles determined by laser granulometry. Milk samples were diluted with 20 mM imidazole–HCl buffer pH 6.6, containing 5 mM $CaCl_2$ and 50 mM NaCl. The accuracy of the particle size determination was ± 10 nm. Complete conditions of high-pressure treatment and sample storage are described in the legend of Fig. 1.

250 MPa carried out at 4, 20 and 40°C on native phosphocaseinate suspension induced the same unexpected variations in the average diameter of particles as those observed with milk samples (190, 160, 185 and 285 nm for unpressurized and pressurized native phosphocaseinate suspensions at 4, 20 and 40°C). High-pressure treatment of 450 and 600 MPa at 4, 20 and 40°C on milk samples led to similar decreases in average diameters of particles determined by laser granulometry. The decreases in the average diameter of particles after high-pressure treatment of milk samples at 20°C are in accordance with the results of Buccheim and Prokopeck (1992) using electron microscopy and with those of Desobry-Banon *et al.* (1994) using laser granulometry.

The effects of high-pressure treatment in combination with temperature on hydration of the ultracentrifuged pellet are illustrated in Fig. 5. High-pressure treatment caused a substantial increase in hydration from 2.0 to 2.4 g water g^{-1} dry pellet. At 4, 20 and 40°C, these increases were similar. Then, above 250 MPa and up to 600 MPa, hydration was constant (about 2.4–2.5 g water g^{-1} dry pellet), except for the high-pressure treatment of 600 MPa at 40°C where we observed a slight decrease in hydration. These increases in hydration were caused by pressure-induced ionization, changes in amino acid side-chains and peptide bonds resulting from solvent exposure, and diffusion of water into cavities located in the hydrophobic core (Masson, 1992; Johnston *et al.*, 1992). Moreover, these increases in hydration in relation to the particle size decreases (Fig. 3) are in good agreement with the results of Anema and Creamer (1993), which indicated that smaller particles have greater solvation.

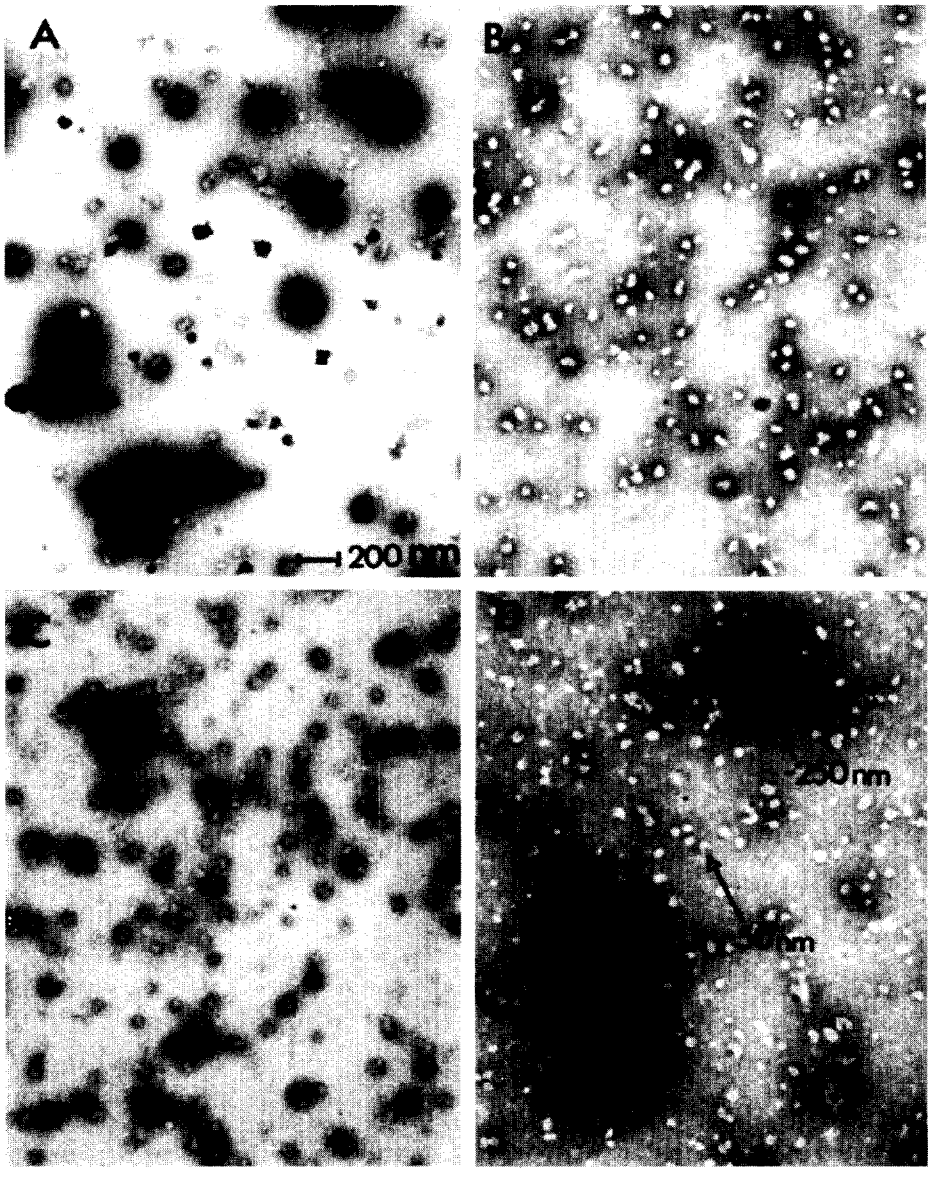

Fig. 4. Combined effects of temperature and high-pressure treatment on the size of casein micelles negatively stained by uranyl acetate and observed by transmission electron microscopy. A, B, C and D correspond to unpressurized and pressurized milk samples (250 MPa) at 4, 20 and 40°C respectively. Complete conditions of high-pressure treatment and sample storage are described in the legend of Fig. 1.

The shape of the plots of soluble calcium (Fig. 6(A)) and phosphorus (Fig. 6(B)) concentrations against high-pressure treatment suggested that, after pressurization of milk at 4, 20 or 40°C, slight solubilization of calcium and phosphorus occurred. No temperature effect was observed. Thus, between 0 and 250 MPa, significant solubilization (about 20 and 25 mg kg^{-1} of calcium and phosphorus, respectively) was observed. Similar results were obtained after pressurization of native phosphocaseinate suspension in ultrafiltrate under the same conditions (results not shown). With milk samples, above 250 MPa and up to 600 MPa, no supplementary calcium and phosphorus solubilization was observed. As the solubilizations of calcium and phosphorus were quantitatively similar, it can be suggested that these elements both came from colloidal calcium phosphate. Moreover, as the soluble casein concentration was unmodified after high-pressure treatment (because no chromatographic peak for caseins was detected in the aqueous phases of milk; results not shown), it can be concluded that the slight solubilization of calcium and phosphorus was not related to the solubilization of an individual casein.

Effects of high-pressure treatment in combination with temperature on whey proteins in milk samples

High-pressure treatment of 250 MPa at 4, 20 or 40°C induced similar decreases in the non-casein protein content (Fig. 7). When the high-pressure treatment was increased to 600 MPa, the non-casein protein content decreased more rapidly as the temperature increased. As observed with heat treatment (Ferron-Baumy *et al.*, 1991) and also with high-pressure treatment (Johnston *et al.*, 1992), these decreases in non-casein protein content were related to an alteration of the whey proteins. Interaction between β-lactoglobulin and κ-casein has been reported after heat treatment (Jang & Swaisgood, 1990; Walstra, 1990). One of the consequences is an increase in rennet clotting time (Ferron-Baumy *et al.*, 1991). On the other hand, after high-pressure treatment,

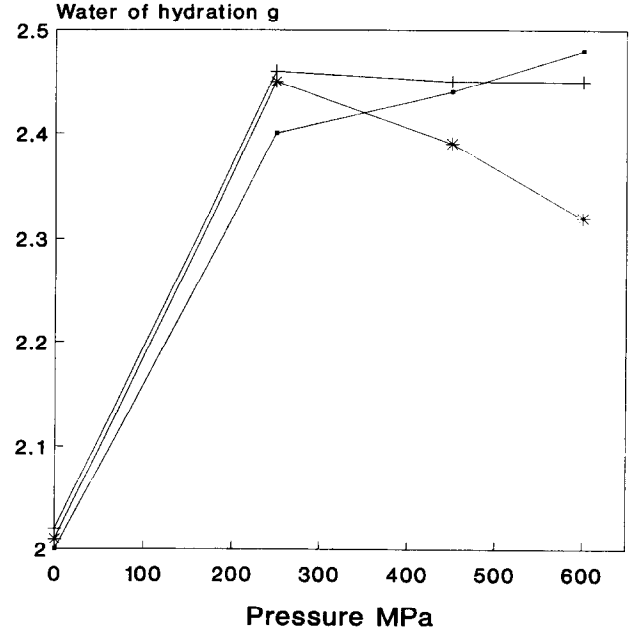

Fig. 5. Combined effects of temperature and high-pressure treatment on pellet hydration. The pellets were obtained by centrifugation at 77 000*g* for 2 h, and then weighed and dried at 103°C for 7 h. The difference between the weight before and after drying, expressed as g water g^{-1} dry pellet was taken as the water of hydration. The accuracy of the determination of the hydration value was ±0.1 g water g^{-1} pellet. Complete conditions of high-pressure treatment and sample storage are described in the legend of Fig. 1.

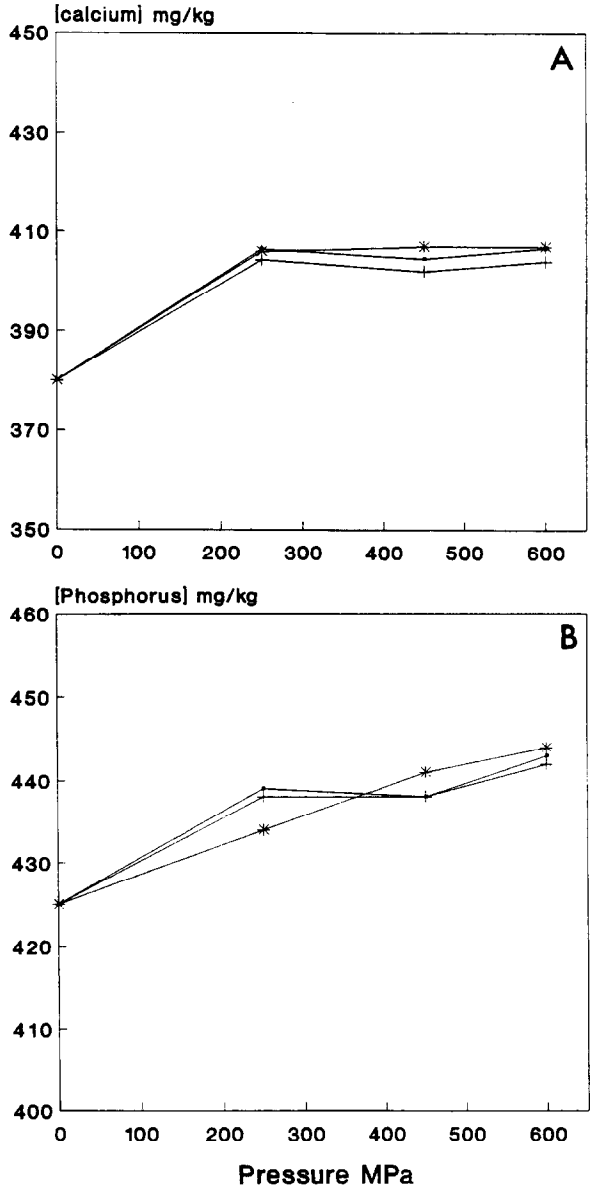

Fig. 6. Combined effects of temperature and high-pressure treatment on the calcium (A) and phosphorus (B) concentrations in the aqueous phases of milk samples. These concentrations were evaluated in the supernatant obtained by centrifugation at 77 000*g* for 2 h. The accuracy of the calcium and phosphorus determination was ±2%. Complete conditions of high-pressure treatment and sample storage are described in the legend of Fig. 1.

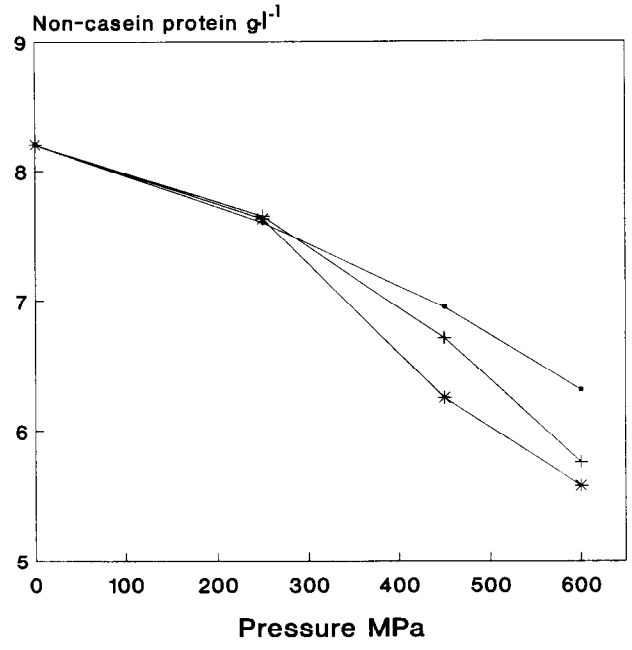

Fig. 7. Combined effects of temperature and high-pressure treatment on non-casein protein contents. The accuracy of the non-casein protein determination was ±0.1 g litre^{-1}. Complete conditions of high-pressure treatment and sample storage are described in the legend of Fig. 1.

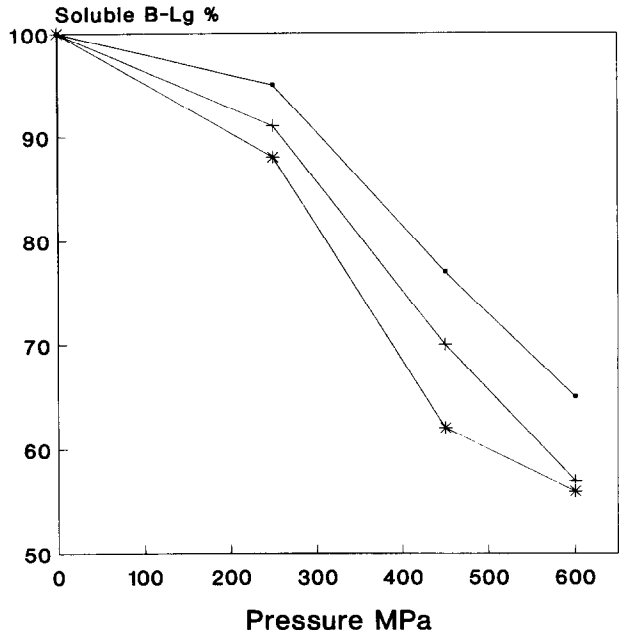

Fig. 8. Combined effects of temperature and high-pressure treatment on the percentage of β-lactoglobulin remaining in the supernatant obtained by centrifugation at 77 000*g* for 2 h. 100% corresponds to the β-lactoglobulin concentration in the supernatant of unpressurized milk. The accuracy of the β-lactoglobulin determination was ±5%. Complete conditions of high-pressure treatment and sample storage are described in the legend of Fig. 1.

a decrease in rennet clotting time was observed (Shibauchi *et al.*, 1992; Desobry-Banon *et al.*, 1994). This result suggested that an association between β-lactoglobulin and κ-casein, as observed after heat treatment, was unlikely. Whey proteins were probably self-aggregated and not covalently associated to caseins. However, from this analysis of soluble nitrogen at pH 4.6, the altered whey proteins were not identified. It should be noted that, at the same time, the non-protein nitrogen contents were unmodified and constant (about 0.337 g litre^{-1} nitrogen), indicating that there was no proteolysis of caseins and whey proteins.

The effects of high-pressure treatment and temperature on the percentage of α-lactalbumin and β-lactoglobulin remaining in the supernatants of different milk samples after centrifugation at 77 000*g* for 2 h were determined by GP-HPLC. In all cases, the percentage of α-lactalbumin was higher than 95%, while the percentage of β-lactoglobulin decreased as applied high pressure increased (Fig. 8). Moreover, the decrease in percentage of β-lactoglobulin was slightly enhanced as the temperature increased. β-Lactoglobulin, at milk pH, is not denatured in the temperature range 4–40°C. Thus, this decrease, which probably corresponds to denaturation of β-lactoglobulin, would be mainly due to the high-pressure treatment. Since the percentage of β-lactoglobulin decreased alone, no association between α-lactalbumin and β-lactoglobulin existed in the ultracentrifugation pellet. This difference in denaturation between α-lactalbumin and β-lactoglobulin was also observed by Nakamura *et al.* (1993) in 10% whey protein concentrate. It could be caused by the difference in the number of disulphide bonds (β-lactoglobulin has two disulphide bonds and α-lactalbumin has four). The resistance to high pressure of α-lactalbumin was probably due to its more rigid molecular structure. With pure solution, a conformational change in β-lactoglobulin during and after high-pressure treatment inducing an irreversible unfolding and aggregation, with formation of intermolecular S–S bonds, was observed (Dufour *et al.*, 1994; Dumay *et al.*, 1994; Futenberger *et al.*, 1995). Moreover, the specific proteolysis of β-lactoglobulin (in whey protein concentrate) by thermolysin, trypsin or pepsin was enhanced under pressure, probably as a result of changes in the structure of β-lactoglobulin (Dufour *et al.*, 1992; Stapelfeldt *et al.*, 1996). From these results, an irreversible pressure-induced denaturation of β-lactoglobulin in milk samples can be proposed. As regards bovine serum albumin, it seems that no major alteration to its secondary structure occurs (Hayakawa *et al.*, 1992).

CONCLUSION

From these results, we have described more fully the effects of high-pressure treament on the physicochemical characteristics of skim milk. Thus, we have confirmed that high-pressure treatment induces an irreversible disintegration of casein micelles into smaller

particles and an increase in protein hydrophobicity. Moreover, we have demonstrated an increase in casein micelle hydration, slight phosphorus and calcium solubilization and β-lactoglobulin denaturation. These changes were related to pressure-induced changes in the hydrogen bonds and in the hydrophobic and electrostatic interactions, which are responsible for maintaining the structural integrity of micelles and of the conformation of globular proteins.

On the other hand, these results showed that it is possible to affect differently the physicochemical characteristics of milk by combination of temperature (4, 20 and 40°C) and high-pressure treatment (250, 450 and 600 MPa). The effects of temperature and of high pressure on protein equilibria were not easy to follow or to interpret. However, knowing that the combination of temperature with high-pressure treatment changes the physicochemical characteristics of milk by acting on the mineral balance, molecular associations and single peptide chain proteins (Walstra & Jenness, 1984; Weber, 1992), different hypotheses can be offered on the effect of temperature at 250 MPa. First, a temperature-dependent dissociation of casein micelles under high pressure followed by different kinds of reassociation during the release of pressure can be suggested. Second, we can propose a dissociation of casein micelles under high pressure followed by a temperature-dependent reassociation during the release of pressure. Third, a combination of these two hypotheses, i.e. a dissociation which depends on the temperature under high pressure and a reassociation also depending on the temperature during the release of pressure, could occur. Thus, after high-pressure treatment of 250 MPa at 40°C, association of casein molecules into clusters could take place. A fourth hypothesis, suggesting that, under these conditions (250 MPa, 40°C), non-covalent interactions between denatured whey proteins and modified casein micelles could exist and that, under other conditions of high-pressure treatment and temperatures, these associations would not be favourable, seems unlikely because similar results were obtained with milk and whey protein-free milk. Further work is therefore needed to clarify the mechanism of high pressure as a function of temperature. Moreover, as the physicochemical characteristics of these milk samples were very dependent on the high pressure and temperature applied, it would be interesting in further studies to test the functional effects such as emulsifying and foaming properties.

ACKNOWLEDGEMENTS

This work was supported by grants from Région Bretagne. Electron microscopy observations were performed at the Centre de microscopie électronique à transmission de l'Université de Rennes I (METUR), Département de Métabio.

REFERENCES

Anema, S. G. & Creamer, L. K. (1993). Effect of the A and B variants of both α_{s1}- and κ-casein on bovine casein micelle solvation and κ-casein content. *J. Dairy Res.*, **60**, 505–516.

Bonomi, F., Iametti, S., Pagliarini, E. & Peri, C. (1988). A spectrofluorimetric approach to the estimation of the surface hydrophobicity modifications in milk proteins upon thermal treatment. *Milchwissenschaft*, **43**, 281–285.

Brulé, G., Maubois, J. L. & Fauquant, J. (1974). Etude de la teneur en éléments minéraux des produits obtenus lors de l'ultrafiltration du lait sur membrane. *Lait*, **54**, 600–615.

Buccheim, W. & Prokopeck, D. (1992). Die Hochdruckbehandlung: ein alternatives Verfahren zur Haltbarmachung und Bearteitung von Milch und anderen Lebensmitteln. *Dtsch Milchwirtsch.*, **43**, 1374–1376.

Carter, J., Knox, D. & Rosenberg, A. (1978). Pressure effects on folded proteins in solution: hydrogen exchange at elevated pressures. *J. Biol. Chem.*, **253**, 1947–1953.

Cheftel, J. C. (1992). Effects of high hydrostatic pressure on food constituents: an overview. In *High Pressure and Biotechnology*, eds C. Balny, R. Hayashi, K. Heremans & P. Masson. Colloq. INSERM. John Libbey Eurotext Ltd, pp. 195–209.

Commission Internationale de l'Éclairage (1971). Colour measurement. CIE Document No. 15.

Desobry-Banon, S., Richard, F. & Hardy, J. (1994). Study of acid and rennet coagulation of high pressurised milk. *J. Dairy Sci.*, **77**, 3267–3274.

Dufour, E., Hervé, G. and Haertlé, T. (1992). Limited proteolysis of bovin β-lactoglobulin with thermolysin under high pressure. In *High Pressure and Biotechnology*, eds C. Balny, R. Hayashi, K. Heremans & P. Masson. Colloq. INSERM. John Libbey Eurotext Ltd, pp. 147–150.

Dufour, E., Hui Bon Hoa, G. & Haertlé, T. (1994). High-pressure effects on β-lactoglobulin interactions with ligands studied by fluorescence. *Biochim. Biophys. Acta*, **1206**, 166–172.

Dumay, E. M., Kalichevsky, M. T. & Cheftel, J. C. (1994). High-pressure unfolding and aggregation of β-lactoglobulin and the baroprotective effects of sucrose. *J. Agric. Food Chem.*, **42**, 1861–1868.

Ferron-Baumy, C., Maubois, J. L., Garric, G. & Quiblier, J. P. (1991). Coagulation présure du lait et des rétentats d'ultrafiltration. Effets de divers traitements thermiques. *Lait*, **71**, 423–434.

Futenberger, S., Duamy, E. & Cheftel, J. C. (1995). Pressure-induced aggregation of β-lactoglobulin in pH 7.0 buffers. *Lebensm. Wiss. Technol.*, **28**, 410–418.

Gross, M. & Jaenicke, R. (1994). Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes. *Eur. J. Biochem.*, **221**, 617–630.

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

International Dairy Federation (1987). Determination of total phosphorus content (photometric method). International Standard FIL-IDF 33C.

Jang, H. D. & Swaisgood, H. E. (1990). Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. *J. Dairy Sci.*, **73**, 900–904.

Johnston, D. E. & Murphy, R. J. (1992). Effects of some calcium-chelating agents on the physical properties of acid-set milk gels. *J. Dairy Res.*, **59**, 197–208.

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

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

Lee, S. K., Anema, S. G., Schrader, K. & Buccheim, W. (1996). Effect of high hydrostatic pressure on Ca-caseinate systems. *Milchwissenschaft*, **51**, 17–20.

Léonil, J., Mollé, D., Gaucheron, F., Arpino, P., Guenot, P. & Maubois, J. L. (1995). Analysis of major bovine milk proteins by on-line high-performance liquid chromatography and electrospray ionization–mass spectrometry. *Lait*, **75**, 193–210.

Masson, P. (1992). Pressure denaturation of proteins. In *High Pressure and Biotechnology*, eds C. Balny, R. Hayashi, K. Heremans & P. Masson. Colloq. INSERM. John Libbey Eurotext Ltd., pp. 89–99.

Nakamura, T., Sado, H. & Syukunobe, Y. (1993). Production of low antigenic whey protein hydrolysates by enzymatic hydrolysis and denaturation with high pressure. *Milchwissenschaft*, **48**, 141–145.

Penniston, J. T. (1971). High hydrostatic pressure and enzymatic activity: inhibition of multimeric enzymes by dissociation. *Arch. Biochem. Biophys.*, **142**, 322–332.

Pierre, A., Michel, F. & Le Graet, Y. (1995). Variation in size of goat milk casein micelles related to casein genotype. *Lait*, **75**, 489–502.

Ruan, K. & Weber, G. (1989). Hysteresis and conformational drift of pressure-dissociated glyceraldehyde phosphate dehydrogenase. *Biochemistry*, **28**, 2144–2153.

Schuck, P., Piot, M., Méjean, S., Le Graet, Y., Fauquant, J., Brulé, G. & Maubois, J. L. (1994). Deshydratation par atomisation de phosphocaseinate natif obtenu par microfiltration sur membrane. *Lait*, **74**, 375–388.

Shibauchi, Y., Yamamoto, H. & Sagara, Y. (1992). Conformational change of casein micelles by high pressure treatment. In *High Pressure and Biotechnology*, eds C. Balny, R. Hayashi, K. Heremans & P. Masson. Colloq. INSERM. John Libbey Eurotext Ltd., pp. 239–242.

Silva, J. L. & Weber, G. (1988). Pressure-induced dissociation of brome mosaic virus. *J. Mol. Biol.*, **199**, 149–159.

Silva, J. L., Miles, E. W. & Weber, G. (1986). Pressure dissociation and conformational drift of the β_2 dimer tryptophan synthase. *Biochemistry*, **25**, 5781–5786.

Stapelfeldt, H., Petersen, P. H., Kristiansen, K. R., Qvist, K. B. & Skibsted, L. H. (1996). Effect of high hydrostatic pressure on the enzymic hydrolysis of β-lactoglobulin B by trypsin, thermolysin and pepsin. *J. Dairy Res.*, **63**, 111–118.

Tausher, B. (1995). Pasteurization of food by hydrostatic high pressure: chemical aspects. *Z. Lebensm. Unters. Forsch.*, **200**, 3–13.

Walstra, P. (1990). On the stability of casein micelles. *J. Dairy Sci.*, **73**, 1965–1979.

Walstra, P. & Jenness, R. (1984). *Dairy Chemistry and Physics*. Wiley, New York.

Weber, G. (1992). *Protein Interactions*. Routledge, Chapman & Hall, pp. 235–270.