Journal of Dairy Research (2004) **71** 489–495. © *Proprietors of Journal of Dairy Research 2004*
DOI: 10.1017/S0022029904000500 *Printed in the United Kingdom*

High pressure-induced denaturation of α-lactalbumin and β-lactoglobulin in bovine milk and whey: a possible mechanism

Thom Huppertz, Patrick F Fox and Alan L Kelly*

Department of Food and Nutritional Sciences, University College Cork, Ireland

Received 18 August 2003 and accepted for publication 22 November 2003

In this study, high pressure (HP)-induced denaturation of α-lactalbumin (α-la) and β-lactoglobulin (β-lg) in dairy systems was examined. In both milk and whey, β-lg was less baroresistant than α-la; both proteins were considerably more resistant to HP-induced denaturation in whey than in milk. HP-induced denaturation of α-la and β-lg increased with increasing proportion of milk in mixtures of milk and whey. Addition of a sulphydryl-oxidising agent, KIO_3, to milk or whey increased HP-induced denaturation of β-lg, but reduced the denaturation of α-la. Denaturation of both α-la and β-lg was prevented by adding a sulphydryl-blocking agent, N-ethylmaleimide, to milk or whey prior to HP treatment, highlighting the crucial role of sulphydryl-disulphide interchange reactions in HP-induced denaturation of α-la and β-lg. Removal of colloidal calcium phosphate from milk also reduced HP-induced denaturation of α-la and β-lg significantly. The higher level of HP-induced denaturation of α-la and β-lg in milk than in whey may be the result of the abscence of the casein micelles and colloidal calcium phosphate from whey, which facilitate HP-induced denaturation of α-la and β-lg in milk.

Keywords: High pressure, milk, whey, α-lactalbumin, β-lactoglobulin, whey protein denaturation.

Denaturation of a protein is any modification in its secondary, tertiary or quaternary conformation that is not accompanied by the rupture of peptide bonds involved in its primary structure; the final conformation of a protein after denaturation can be that of a totally- or partially-unfolded polypeptide. In their native state, proteins are stabilised by covalent bonds (including disulphide bridges), electrostatic interactions (ion pairs, polar groups), hydrogen bonds and hydrophobic interactions. One of the most extensively studied mechanisms for denaturing proteins in food systems is probably thermal treatment. However, high pressure (HP) treatment also affects protein conformation, in a manner depending on the protein itself, its environment, and the applied pressure, temperature and duration of treatment.

The primary structure of proteins remains intact on HP treatment (Gross & Jaenicke, 1994; Mozhaev et al. 1994). Hydrogen bonds, which stabilise the secondary structure, are enhanced at low pressures and ruptured only at very high pressures (Hendrickx et al. 1998). Significant changes in the tertiary structure of proteins, which is maintained chiefly by hydrophobic and ionic interactions, are observed >200 MPa (Hendrickx et al. 1998). Ionic bonds in aqueous solution are strongly destabilised by pressure (Gross & Jaenicke, 1994), as are hydrophobic interactions between aliphatic groups (Heremans, 1982; Mozhaev et al. 1994). It is clear that HP treatment at moderate temperatures disrupts only relatively weak bonds, such as hydrogen, hydrophobic and ionic bonds (Hendrickx et al. 1998).

Of particular interest for milk and dairy products is HP-induced denaturation of the major whey proteins, α-lactalbumin (α-la) and β-lactoglobulin (β-lg). Studies on milk have shown that α-la is more baroresistant than β-lg (López-Fandiño et al. 1996; Felipe et al. 1997; López-Fandiño & Olano, 1998; Garcia-Risco et al. 2000; Scollard et al. 2000; Huppertz et al. 2004). On HP treatment of milk, denatured β-lg may form small aggregates (Felipe et al. 1997) or interact with casein micelles (Needs et al. 2000a; Scollard et al. 2000). Huppertz et al. (2004) showed that the majority of denatured β-lg in HP-treated milk was co-sedimentable with the casein micelles. Interactions between β-lg and casein micelles are probably due to disulphide bonding (Needs et al. 2000b) with κ-casein (López-Fandiño et al. 1997; López-Fandiño & Olano, 1998), but the exact mechanism for these processes needs further clarification.

*For correspondence; e-mail: a.kelly@ucc.ie

HP-induced denaturation of α-la and β-lg has been studied in considerably more detail in aqueous solutions and buffers than in milk. In such systems, it has been shown that high pressure promotes unfolding and aggregation of β-lg through sulphydryl-disulphide interchange reactions (Dumay et al. 1994; Funtenberg et al. 1997; Van Camp et al. 1997). HP-induced aggregation of β-lg can be prevented by addition of sulphydryl-blocking (Tanaka et al. 1996; Funtenberger et al. 1997) or disulphide-reducing agents (Funtenberger et al. 1997) to the solutions prior to HP treatment. HP-induced aggregation of β-lg in buffer was enhanced by higher calcium concentrations (Van Camp et al. 1997). Dumay et al. (1994) and Van Camp et al. (1997) suggested that HP-induced aggregation of β-lg may be partially reversible on subsequent storage. Mixed aggregates of denatured α-la and β-lg have been shown in HP-treated whey protein solutions in buffer (Jegouic et al. 1997).

This study focussed on HP-induced denaturation of α-la and β-lg in whey, milk and mixtures thereof, and on the influence of sulphydryl-modifying agents and calcium on the denaturation of α-la and β-lg in these media, to further elucidate the mechanism for HP-induced denaturation of α-la and β-lg in milk and whey.

Materials and Methods

Milk and whey supply

Raw whole bovine milk, obtained from a local dairy (CMP Dairies, Cork), was skimmed by centrifugation at 2000 ***g*** at 20 °C for 20 min, followed by filtration of the subnatant through glass wool to remove fat particles. Sodium azide (0·5 g/l) was added to the skimmed milk to prevent microbial growth and its pH was adjusted to 6·6, using 2·0 M-HCl.

Rennet (Maxiren 180, DSM Food Specialties, Delft, The Netherlands) was added to raw skim milk at 31 °C at a level of 400 μl/l and the milk was left to coagulate at 31 °C for 45 min. The coagulum was cut and cooked to 40 °C over 30 min, to enhance curd syneresis, and the curds and whey separated by filtration through cheesecloth. The whey was centrifuged at 3000 ***g*** at 20 °C for 15 min and filtered through glass wool to remove curd particles. The pH of the whey was adjusted to 6·6, using 2·0 M-NaOH or 2·0 M-HCl.

Preparation of mixtures of milk and whey

To study the influence of casein micelles on HP-induced denaturation of α-la and β-lg, mixtures of milk and whey were prepared. Residual coagulant in whey was inhibited by adding a stock solution (1 mg/ml) of pepstatin A (Sigma Corp., St. Louis, MO, USA) in a 90 : 10 (v/v) mixture of ethanol-acetic acid to whey, at a level of 34·5 ml/l, i.e. a final pepstatin A concentration of 50 μM; preliminary experiments had shown that 50 μM pepstatin A in milk was sufficient to completely inhibit any caseinolytic activity of residual coagulant for ⩾24 h. The pH of the whey containing pepstatin A was readjusted to 6·6 using 2·0 M-NaOH. Milk and whey were then mixed at ratios of 0 : 100, 10 : 90, 25 : 75, 50 : 50 and 100 : 0.

Addition of sulphydryl-modifying agents to whey and milk

To study the role of sulphydryl groups in HP-induced denaturation of α-la and β-lg, a sulphydryl blocking agent, N-ethylmaleimide (NEM), was added to milk or whey prior to HP treatment. NEM was dissolved (20 mg/ml) in N,N-dimethylformamide (DMF) and 16·7 ml/l of this stock solution were added to whey or milk, to reach a final concentration of 2·67 mmol/l i.e. an estimated 8-fold molar excess with respect to the level of free sulphydryl-groups in β-lg in milk (~0·33 mmol/l). In control experiments, an equivalent volume of DMF, without NEM, was added to a sample of milk or whey. In separate experiments, a sulphydryl oxidising agent, KIO_3 (0·1 mM), was added to milk or whey prior to HP treatment. All samples were incubated with NEM, DMF or KIO_3 at 5 °C for 24 h prior to treatment.

Preparation of colloidal calcium phosphate-free skim milk

Colloidal calcium phosphate (CCP)-free skim milk was prepared using an adaptation from the method described by Pyne & McGann (1960). The pH of raw skim milk was adjusted to 4·6 at 5 °C with 2·0 M-HCl and the acidified milk was dialysed for 48 h against 2 × 20 volumes of raw skim milk at 5 °C. Finally, the pH of the dialysed milk was readjusted to 6·6 at 20 °C with 2·0 M-NaOH. This process removes virtually all colloidal phosphate from milk and most of colloidal calcium (Pyne & McGann, 1960); the remainder of the colloidal calcium is thought to be bound to the caseins (Pyne, 1962).

High pressure and thermal treatment of milk and whey

Samples (1·5–25 ml) were vacuum-packaged in polyethylene bags (100 × 150 mm; Miller Pack Ltd., Finglas, Dublin 11, Ireland) or placed in polypropylene tubes (capacity 1·5 ml; Sarstedt, Nürmbrecht, Germany), leaving no headspace. Packaged samples were placed in polyethylene bags containing ~100 ml water, vacuum-packaged and stored at 20 °C for at least 30 min, but no longer than 4 h.

HP treatment was performed using a Stansted Fluid Power Iso-Lab 900 High Pressure Food Processor (Stansted Fluid Power, Stansted, Essex, UK) with a 90 : 10 mixture of ethanol and castor oil as the pressure-transmitting medium. This pressure vessel has a 2 l capacity and an internal diameter of 100 mm. Pressure was increased at a rate of 300 MPa/min to a value in the range 100–800 MPa and

https://doi.org/10.1017/S0022029904000500 Published online by Cambridge University Press

maintained at the desired pressure for 30 or 60 min, after which pressure was released at a rate of 300 MPa/min. The temperature of the vessel of the HP unit was thermostatically controlled at 20°C throughout treatment. Due to compressive heating, increases in the temperature of the processing fluid, by up to a maximum of 15 °C at 800 MPa, were observed; increases in the temperature of the processing fluid were transient, and the set temperature ±1 °C was re-attained within 10 min of the start of treatment.

For comparison, samples of milk or whey in Eppendorf tubes were heated at 90 °C for 30 min using an Eppendorf thermomixer (Eppendorf AG, Hamburg, Germany), with continuous mixing at 600 rpm.

Measurement of denaturation of α-lactalbumin or β-lactoglobulin

Denaturation of α-la and β-lg was estimated by determining the level of residual native α-la or β-lg in milk or whey by reversed-phase high performance liquid chromatography (RP-HPLC). The pH 4·6-soluble fraction of milk or whey, prepared within 15 min of the end of HP treatment, as described by Huppertz et al. (2004), was diluted 1 : 9 with HPLC solvent A (0·1 %, v/v, trifluoroacetic acid [TFA] in deionised water) and filtered through 0·45 µm Millex filters (Millipore Corporation, Bedford, MA, USA). Preparation of the pH 4·6-soluble phase of milk at a set time after HP treatment, 15 min in this study, is necessary because HP-induced denaturation of β-lg may be partially reversible (Dumay et al. 1994; Van Camp et al. 1997).

RP-HPLC was performed using a Waters HPLC system equipped with a Degasys DG-2410 online degassing unit (Sanwa Tsusho Co, Minato-ku Tokyo, Japan), as described by Huppertz et al. (2004). Samples (100 µl) were applied to the column and eluted with 100% solvent A for 5 min, followed by a linear gradient to 45% solvent B (0·1 %, v/v, TFA in far UV HPLC-grade acetonitrile; Labscan Ltd., Dublin, Ireland) over 5 min; the eluent was then adjusted in a linear gradient to 60% solvent B over 15 min, maintained at this level for 15 min, increased in a linear gradient to 95% solvent B over 5 min, and maintained at that level for 3 min. The eluent was then readjusted to 100% solvent A over 2 min and the system was allowed to equilibrate for 25 min before injection of the next sample. The flow rate was maintained at 0·75 ml/min and the eluate was monitored at 214 nm. Peaks corresponding to α-la and β-lg were identified and levels of native α-la or β-lg calculated as described by Huppertz et al. (2004).

Statistical analysis

All results presented are means from three independent experiments on independent samples of milk or whey. Statistical analysis was performed using a randomised block design, using Minitab version 12 (Minitab Ltd., Coventry, UK). The influence of the level of milk in whey, the addition of NEM or KIO_3 to milk or whey, or the removal of CCP from milk on thermal or HP-induced denaturation of α-la and β-lg was examined using the General Linear Model technique, with Tukey's pairwise comparisons at a 95% confidence level.

Results

High pressure-induced denaturation of α-la and β-lg in mixtures of milk and whey

In milk, whey and mixtures thereof, denaturation of α-la was not observed $\leqslant$400 MPa (data not shown). After treatment at 600 MPa for 30 or 60 min, little difference in the level of denatured α-la was observed between whey samples containing 0, 10 or 25% milk, whereas the level of denatured α-la in whey containing 50% milk or in 100% milk was slightly higher than in whey samples containing lower levels of milk. After treatment at 800 MPa, the level of denatured α-la was significantly ($P<0·05$) higher in milk than in whey samples containing 0, 10, 25 or 50% milk (Table 1). Denaturation (>10%) of β-lg in milk or whey occurred on treatment at $\geqslant$200 or $\geqslant$400 MPa, respectively (Table 1). On treatment for 30 min at $\geqslant$400 MPa or 60 min at 400 MPa, the level of denatured β-lg increased in a linear manner (R^2 value >98% for correlations; all $P<0·001$) with increasing level of milk in the mixture.

Influence of sulphydryl-modifying agents and calcium on high pressure-induced denaturation of α-la and β-lg in milk and whey

HP-induced denaturation of α-la and β-lg was influenced significantly by addition of NEM or KIO_3 to milk or whey prior to treatment. DMF, in which the NEM was dissolved, did not influence HP-induced or thermal denaturation of α-la and β-lg (data not shown). In milk or whey containing NEM, very little denaturation of α-la or β-lg occurred on HP treatment at up to 800 MPa (Table 2). NEM also significantly ($P<0·05$) reduced, but did not prevent, denaturation of α-la and β-lg in milk or whey on heating at 90 °C for 30 min (Table 2).

The presence of 0·1 mmol L^{-1} KIO_3 in milk significantly ($P<0·05$) reduced the extent of HP-induced denaturation of α-la, but significantly increased the denaturation of β-lg in milk and whey (Table 2); KIO_3 also significantly reduced the extent of thermal denaturation of α-la in milk or whey (Table 2).

In control and CCP-free milk, β-lg or α-la were denatured on treatment at $\geqslant$200 or $\geqslant$600 MPa, respectively (Table 3), but the level of denaturation of both proteins was significantly ($P<0·05$) lower in CCP-free milk than in the control. After heating at 90 °C for 30 min, similar levels of denatured α-la and β-lg were measured in control and CCP-free milk (Table 3).

https://doi.org/10.1017/S0022029904000500 Published online by Cambridge University Press

Table 1. Effect of high pressure treatment at 200–800 MPa at 20 °C for 30 or 60 min on the level of denatured α-lactalbumin (α-la) or β-lactoglobulin (β-lg) in mixtures of milk and rennet whey, expressed as a percentage of the total level of α-la or β-lg for the respective untreated milk: whey mixture.

Values are means of data from triplicate experiments on individual milk and whey samples, ±standard deviation

Pressure (MPa)	Treatment time (min)	0% milk; 100% whey	10% milk; 90% whey	25% milk; 75% whey	50% milk; 50% whey	100% milk; 0% whey
				% denatured α-la		
600	30	13·3±6·1^{a}	14·5±4·1^{a}	16·3±5·5^{ab}	21·8±2·9^{b}	25·6±7·5^{b}
	60	18·2±4·9^{a}	18·0±3·8^{a}	20·4±6·4^{ab}	28·5±1·2^{b}	32·0±6·5^{b}
800	30	16·6±6·9^{a}	16·7±6·2^{a}	17·6±8·7^{a}	18·6±4·0^{a}	36·2±4·1^{b}
	60	17·6±5·1^{a}	18·5±2·8^{a}	22·4±4·2^{ab}	26·4±4·7^{b}	59·5±5·9^{c}
				% denatured β-lg		
200	30	2·8±2·1^{a}	5·6±2·1^{ab}	8·5±4·7^{abc}	12·9±5·9^{bc}	18·5±5·9^{c}
	60	5·8±3·2^{a}	9·0±3·9^{ab}	16·1±4·9^{bc}	21·0±5·9^{cd}	32·8±5·6^{d}
400	30	46·1±3·7^{a}	50·0±6·1^{a}	55·4±6·7^{ab}	66·4±4·9^{b}	81·1±4·7^{c}
	60	55·2±6·8^{a}	62·4±5·8^{ab}	67·1±5·9^{bc}	79·8±6·4^{c}	93·8±3·2^{d}
600	30	60·8±2·8^{a}	64·2±3·6^{a}	72·9±3·3^{b}	80·2±3·4^{c}	95·7±4·1^{d}
	60	66·5±1·6^{a}	72·9±1·6^{b}	79·4±1·9^{c}	90·3±2·5^{d}	98·9±0·4^{e}
800	30	80·1±6·3^{a}	81·2±4·3^{a}	84·2±5·9^{a}	88·7±5·7^{a}	98·8±1·5^{b}
	60	81·2±1·6^{a}	87·1±0·7^{b}	91·1±1·9^{c}	96·5±1·2^{d}	99·9±0·1^{e}

NOTE: no denaturation of α-la occurred on treatment at pressures ⩽400 MPa
^{a,b,c,d,e} Values without a common superscript in a row were significantly different ($P<0·05$)

Table 2. Effect of high pressure treatment at 200–800 MPa at 20 °C for 30 min or heating at 90 °C for 30 min on the denaturation of α-lactalbumin (α-la) or β-lactoglobulin (β-lg) in milk or rennet whey, or in milk or rennet whey containing 334 mg NEM/l or 0·1 mM-KIO_3.

Values are expressed as a percentage of the level of total α-la or β-lg for respective untreated milk or whey samples and are means of data from triplicate experiments on individual milk and rennet whey samples, ±standard deviation

Treatment (30 min)	Milk			Rennet whey		
	Control	+NEM	+KIO_3	Control	+NEM	+KIO_3
			% denatured α-la			
600 MPa	22·9±2·3^{a}	1·4±2·4^{b}	5·6±1·9^{b}	6·3±5·5^{X}	0·2±5·6^{X}	1·3±4·0^{X}
800 MPa	53·6±5·4^{a}	4·9±5·3^{b}	9·2±3·1^{b}	8·1±3·4^{X}	0·8±4·8^{Y}	1·5±2·3^{Y}
90 °C	91·7±1·4^{a}	13·4±1·4^{b}	20·0±5·4^{c}	94·6±0·5^{X}	24·8±1·9^{Y}	56·8±0·1^{Z}
			% denatured β-lg			
200 MPa	16·2±1·4^{a}	0·2±4·4^{b}	54·6±5·7^{c}	0·9±1·5^{X}	0·6±2·3^{X}	21·0±3·6^{Y}
400 MPa	86·9±2·9^{a}	0·3±4·5^{b}	96·1±1·8^{c}	24·8±4·4^{X}	0·6±3·0^{Y}	69·2±6·0^{Z}
600 MPa	97·6±0·3^{a}	2·5±3·4^{b}	98·0±1·1^{c}	58·0±7·6^{X}	1·8±2·2^{Y}	74·1±5·2^{Z}
800 MPa	99·7±0·2^{a}	4·9±2·5^{b}	98·2±0·9^{a}	87·5±3·9^{X}	1·4±6·4^{Y}	79·3±1·3^{Z}
90 °C	100·0±0·0^{a}	82·4±0·5^{b}	100·0±0·0^{a}	100·0±0·0^{X}	59·6±1·7^{Y}	99·5±0·0^{X}

NOTE: no denaturation of α-la occurred on treatment at pressures ⩽400 MPa
^{a,b,c} Values for milk without a common lower-case superscript in a row were significantly different ($P<0·05$)
^{X,Y,Z} Values for rennet whey without a common upper-case superscript in a row were significantly different ($P<0·05$)

Discussion

The higher stability of α-la, compared with β-lg, to HP-induced denaturation in milk and whey (Tables 1–3), which is consistent with previous observations on milk (López-Fandiño et al. 1996; Felipe et al. 1997; López-Fandiño & Olano, 1998; Garcia-Risco et al. 2000; Scollard et al. 2000; Huppertz et al. 2004), is considered to be due to the higher number of disulphide bonds (4 in α-la, 2 in β-lg) and the presence of a free sulphydryl group in β-lg (Hinrichs et al. 1996; López-Fandiño et al. 1996).

HP treatment caused considerably less denaturation of α-la and β-lg in whey than in milk (Tables 1 & 2). Compared with milk, thermal denaturation of whey proteins is lower in cheese whey (Hillier & Lyster, 1979), acid whey readjusted to pH 6·7 (Law & Leaver, 1999) and, in the case of α-la, in milk ultrafiltrate (Baer et al. 1976). On HP treatment (Table 1), as well as on thermal treatment (Law & Leaver, 1999), denaturation of α-la and β-lg

https://doi.org/10.1017/S0022029904000500 Published online by Cambridge University Press

Table 3. Effect of high pressure treatment at 200–800 MPa at 20 °C for 30 min or heating at 90 °C for 30 min on the level of denatured α-lactalbumin (α-la) or β-lactoglobulin (β-lg) in control or colloidal calcium phosphate-free (CCP-free) skim milk.

Values are expressed as a percentage of total α-la or β-lg in milk and are means of data from triplicate experiments on individual milk samples, ± standard deviation

	% denatured α-la		% denatured β-lg	
Treatment (30 min)	Control milk	CCP-free milk	Control milk	CCP-free milk
200 MPa	0·1 ± 2·3^{a}	0·5 ± 3·0^{a}	16·5 ± 4·0^{X}	10·0 ± 4·4^{X}
400 MPa	2·0 ± 7·1^{a}	1·1 ± 1·7^{a}	89·8 ± 3·2^{X}	61·1 ± 5·4^{Y}
600 MPa	33·2 ± 2·4^{a}	19·1 ± 5·6^{b}	99·0 ± 1·6^{X}	69·2 ± 3·2^{Y}
800 MPa	59·4 ± 4·7^{a}	24·8 ± 6·8^{b}	100·0 ± 0·0^{X}	89·8 ± 1·2^{Y}
90 °C	94·9 ± 1·2^{a}	96·7 ± 3·0^{a}	100·0 ± 0·0^{X}	99·1 ± 1·5^{X}

^{a,b} Values for α-la without a common lower-case superscript in a row were significantly different ($P<0·05$)
^{X,Y} Values for β-lg without a common upper-case superscript in a row were significantly different ($P<0·05$)

increased with increasing level of milk in mixtures of milk and whey. However, thermal denaturation of whey proteins increases markedly with increasing milk content only up to ~30%, with little further effect at higher levels of milk (Law & Leaver, 1999), whereas HP-induced denaturation of whey proteins increased progressively with increasing milk content (Table 1).

Under HP, β-lg unfolds (Tanaka et al. 1996; Moller et al. 1998; Knudsen et al. 2002), resulting in the exposure of its free sulphydryl group (Tanaka et al. 1996; Moller et al. 1998; Stapelfeldt et al. 1999). Unfolded β-lg can interact, through sulphydryl-disulphide interchange reactions, with proteins containing disulphide bonds, e.g. α_{s2}- and κ-caseins (Swaisgood, 2003), α-la (Brew, 2003) and β-lg (Sawyer, 2003). On HP treatment of milk, aggregates of β-lg are formed (Felipe et al. 1997), but most denatured β-lg interacts with casein micelles and the level of β-lg associated with the casein micelles is proportional to the level of denatured β-lg (Huppertz et al. 2004). The lower extent of HP-induced denaturation of β-lg in whey than in milk may be explained by the lower number of molecules available for interaction than in milk, due to the absence of casein micelles from whey.

The inhibitory effect of NEM on thermal denaturation of α-la and β-lg (Table 2) agrees with previous observations on milk (Morr & Josephson, 1968), cheese whey (Morr & Josephson, 1968; Donovan & Mulvihill, 1987a), or solutions of α-la or β-lg in simulated milk ultrafiltrate (Elfagm & Wheelock, 1978) or water (Sawyer, 1968). NEM prevents heat-induced aggregation by inhibiting sulphydryl oxidation and/or sulphydryl-disulphide interchange reactions (Donovan & Mulvihill, 1987a) and a similar effect probably occurs on HP treatment of milk or whey. NEM has previously been shown to inhibit HP-induced denaturation of β-lg in buffers (Tanaka et al. 1996; Funtenberger et al. 1997). Sawyer (1968) reported that when β-lg is heated in the presence of NEM it can aggregate *via* a different mechanism; as HP-induced denaturation of α-la and β-lg did not occur in the presence of NEM (Table 2) it seems that HP-induced denaturation of these proteins occurs only through sulphydryl-disulphide interchange or sulphydryl oxidation reactions. α-La does not have a free sulphydryl group and can undergo sulphydryl-disulphide interchange reactions only with a protein containing a free sulphydryl-group. Aggregates of α-la and β-lg, formed through sulphydryl-disulphide interchange reactions, have been observed in a HP-treated solution of α-la and β-lg in buffer (Jegouic et al. 1997). The fact that HP-induced denaturation of α-la and β-lg did not occur when sulphydryl-disulphide interchange reactions in milk or whey were prevented by addition of NEM (Table 2) suggests that unfolded β-lg and α-la which had not interacted with other proteins may refold to their native conformation on release of pressure.

The presence of KIO_3 increased the stability of α-la but reduced the stability of β-lg to denaturation on thermal treatment of milk (Table 2; Skudder et al. 1981; Enright et al. 1999) and whey (Table 2) or HP treatment of milk and whey (Table 2). KIO_3 may oxidise sulphydryl-groups in proteins as follows (Hird & Yates, 1961):

$$6\,\text{protein}-\text{SH}+\text{IO}_3^- \rightarrow 3\,\text{protein}-\text{S}-\text{S}-\text{protein}+3\text{H}_2\text{O}+\text{I}^- \quad (1)$$

$$\text{Protein}-\text{SH}+\text{IO}_3^- \rightarrow \text{protein}-\text{SO}_3\text{H}+\text{I}^- \quad (2)$$

In this study, reaction (1) may have occurred preferentially, due to the relatively high $-SH:IO_3^-$-ratio, estimated to be ~2 : 1, resulting in increased HP-induced denaturation of β-lg in the presence of KIO_3 (Table 2). Reduced HP-induced denaturation of α-la in the presence of KIO_3 is probably due to the fact that sulphydryl-disulphide interchange reactions of that protein with β-lg cannot occur because its sulphydryl-group is oxidised.

HP-induced denaturation of α-la and β-lg was more extensive in control milk than in CCP-free milk, suggesting that a higher level of calcium enhances denaturation of these proteins, in agreement with observations by Van Camp et al. (1997) in aqueous solutions of whey protein concentrate. Increased thermal denaturation of whey proteins with increasing calcium content has been observed in milk (Table 3), cheese whey (de Rham & Chanton, 1984; Donovan & Mulvihill, 1987b), simulated milk ultrafiltrate (Paulsson & Dejmek, 1990) and acid whey readjusted to neutral pH (Morr & Josephson, 1968). Calcium may neutralise the net negative charge on unfolded whey proteins, reducing intermolecular electrostatic repulsion, and mediating the close approach of other proteins, thus facilitating sulphydryl-disulphide interchange reactions (Donovan & Mulvihill, 1987b). CCP is solubilised by HP, resulting in an increased level of soluble calcium (De la Fuente et al. 1999; Arias et al. 2000), which may contribute to reducing the net-negative charge

https://doi.org/10.1017/S0022029904000500 Published online by Cambridge University Press

on the whey proteins. Since HP-induced solubilisation of CCP cannot occur in CCP-free milk, electrostatic repulsion between unfolded whey proteins and other proteins in CCP-free milk may be higher under HP than in control milk, thereby reducing denaturation of α-la and β-lg (Table 3). The absence of CCP from rennet whey may thus also contribute to the lower extent of HP-induced denaturation observed in this medium, compared with in milk (Tables 1 & 2).

Conclusions

HP-induced denaturation of α-la and β-lg in milk and whey may be through the following mechanism: protein unfolding under HP exposes the free sulphydryl group in β-lg, which, through sulphydryl-disulphide interchange reactions can form aggregates with κ-casein, α-la or β-lg. On release of pressure, unfolded α-la and β-lg that have not interacted with other proteins may refold to their native state. Calcium may facilitate close approach of unfolded whey proteins with other proteins. Thus, the extent of HP-induced denaturation of α-la and β-lg depends on the amount of available unmodified sulphydryl groups as well as on the level of calcium in the medium.

This research was funded by the Food Institutional Research Measure (FIRM) which is administered by the Irish Government under the National Development Plan, 2000–2006. The technical assistance of Therese Uniacke (Department of Food and Nutritional Sciences, University College Cork) was greatly appreciated.

References

Arias M, López-Fandiño R & Olano A 2000 Influence of pH on the effects of high pressure on milk proteins. *Milchwissenschaft* **55** 191–194

Baer A, Oroz M & Blanc B 1976 Serological studies on heat-induced interactions of α-lactalbumin and milk proteins. *Journal of Dairy Research* **43** 419–432

Brew K 2003 α-Lactalbumin. In *Advanced Dairy Chemistry Volume 1: Proteins, 3rd ed.*, pp. 387–421 (Eds PF Fox & PLH McSweeney). New York: Kluwer Academic/Plenum Publishers

De la Fuente MA, Olano A, Casal V & Juarez M 1999 Effects of high pressure and heat treatment on the mineral balance of goats' milk. *Journal of Dairy Research* **66** 65–72

De Rham O & Chanton S 1984 Role of ionic environment in insolubilization of whey protein during heat treatment of whey products. *Journal of Dairy Science* **67** 939–949

Donovan M & Mulvihill DM 1987a Effects of chemical modification and sodium dodecyl sulphate binding on the thermostability of whey proteins. *Irish Journal of Food Science and Technology* **11** 77–85

Donovan M & Mulvihill DM 1987b Thermal denaturation and aggregation of whey proteins. *Irish Journal of Food Science and Technology* **11** 87–100

Dumay EM, Kalichevsky MT & Cheftel JC 1994 High pressure unfolding and aggregation of beta-lactoglobulin and the baroprotective effects of sucrose. *Journal of Agricultural and Food Chemistry* **42** 1861–1868

Elfagm AA & Wheelock JV 1978 Interaction of bovine α-lactalbumin and β-lactoglobulin during heating. *Journal of Dairy Science* **61** 28–32

Enright E, Bland AP, Needs EC & Kelly AL 1999 Proteolysis and physico-chemical changes in milk on storage as affected by UHT treatment, plasmin activity and KIO_3 addition. *International Dairy Journal* **9** 581–591

Felipe X, Capellas M & Law AJR 1997 Comparison of the effects of high-pressure treatment and heat pasteurization on the whey proteins in goats' milk. *Journal of Agricultural and Food Chemistry* **45** 627–631

Funtenberger S, Dumay EM & Cheftel JC 1997 High pressure promotes betalactoglobulin aggregation through SH/S-S interchange reactions. *Journal of Agricultural and Food Chemistry* **45** 912–921

Garcia-Risco MR, Olano A, Ramos M & López-Fandiño R 2000 Micellar changes induced by high pressure. Influence in the proteolytic activity and organoleptic properties of milk. *Journal of Dairy Science* **83** 2184–2189

Gross M & Jaenicke R 1994 Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes. *European Journal of Biochemistry* **221** 617–630

Hendrickx M, Ludikhuyze L, Van den Broek I & Weemaes C 1998 Effects of high pressure on enzymes related to food quality. *Trends in Food Science and Technology* **9** 197–203

Heremans K 1982 High pressure effects on proteins and other biomolecules. *Annual Reviews in Biophysics and Bioengineering* **11** 1–21

Hillier RM & Lyster RLJ 1979 Whey protein denaturation in heated milk and cheese whey. *Journal of Dairy Research* **46** 95–102

Hinrichs J, Rademacher B & Kessler HG 1996 Reaction kinetics of pressure-induced denaturation of whey proteins. *Milchwissenschaft* **51** 504–509

Hird FJR & Yates JR 1961 The oxidation of cysteine, glutathione and thioglyiollate by iodate, bromate, persulfate and air. *Journal of Science of Food and Agriculture* **12** 89–95

Huppertz T, Fox PF & Kelly AL 2004 High pressure treatment of bovine milk: effects on casein micelles and whey proteins. *Journal of Dairy Research* **71** 97–106

Jegouic M, Grinberg VY, Guingant A & Haertle T 1997 Baric oligomerization in α-lactalbumin/β-lactoglobulin mixtures. *Journal of Agricultural and Food Chemistry* **45** 19–22

Keenan TW & Dylewski DP 1995 Intracellular origin of milk lipid globules and the nature and structure of the milk lipid globule membrane. In *Advanced Dairy Chemistry Volume 2: Lipids, 2nd Edition*, pp. 89–130 (Ed. PF Fox). London: Chapman & Hall

Knudsen JC, Otte J, Olsen K & Skibsted LH 2002 Effect of high hydrostatic pressure on the conformation of β-lactoglobulin A as assessed by proteolytic peptide profiling. *International Dairy Journal* **12** 791–803

Law AJR & Leaver J 1999 Factors affecting the heat denaturation of whey proteins in cow's milk. *International Dairy Journal* **9** 407–408

López-Fandiño R & Olano A 1998 Effects of high pressures combined with moderate temperatures on the rennet coagulation properties of milk. *International Dairy Journal* **8** 623–627

López-Fandiño R, Carrascosa AV & Olano A 1996 The effects of high pressure on whey protein denaturation and cheese-making properties of raw milk. *Journal of Dairy Science* **79** 929–936

López-Fandiño R, Ramos M & Olano A 1997 Rennet coagulation of milk subjected to high pressures. *Journal of Agricultural and Food Chemistry* **45** 3233–3237

Moller RE, Stapelfeldt H & Skibsted LH 1998 Thiol reactivity in pressure-unfolded β-lactoglobulin. Antioxidative properties and thermal refolding. *Journal of Agricultural and Food Chemistry* **46** 425–430

Morr CV & Josephson RV 1968 Effect of calcium, N-ethylmaleimide and casein upon heat-induced whey protein aggregation. *Journal of Dairy Science* **51** 1349–1355

Mozhaev VV, Heremans K, Frank J, Masson P & Balny C 1994 Exploiting the effects of high hydrostatic pressure in biotechnological applications. *Trends in Biotechnology* **12** 493–501

Needs EC, Capellas M, Bland AP, Manoj P, MacDougal D & Paul G 2000a Comparison of heat and pressure treatments of skimmed milk, fortified with whey protein concentrate, for set yoghurt preparation: effects on milk proteins and gel structure. *Journal of Dairy Research* **67** 329–348

Needs EC, Stenning RA, Gill AL, Ferragut V & Rich GT 2000b High-pressure treatment of milk: effects on casein micelle structure and on enzymic coagulation. *Journal of Dairy Research* **67** 31–42

Paulson M & Dejmek P 1990 Thermal denaturation of whey proteins in mixtures with caseins studied by differential scanning calorimetry. *Journal of Dairy Science* **73** 590–600

Pyne GT 1962 Reviews of progress of dairy science – some aspects of physical chemistry of salts of milk. *Journal of Dairy Research* **29** 101–130

Pyne GT & McGann TCA 1960 The colloidal phosphate of milk. 2. Influence of citrate. *Journal of Dairy Research* **27** 9–17

Sawyer WH 1968 Heat denaturation of bovine β-lactoglobulins and relevance of disulphide aggregation. *Journal of Dairy Science* **51** 323–329

Sawyer L 2003 β-Lactoglobulin. In *Advanced Dairy Chemistry Volume 1: Proteins, 3rd Edition*, pp. 319–386 (Eds PF Fox & PLH McSweeney). New York: Kluwer Academic/Plenum Publishers

Scollard PG, Beresford TP, Needs EC, Murphy PM & Kelly AL 2000 Plasmin activity, β-lactoglobulin denaturation and proteolysis in high pressure treated milk. *International Dairy Journal* **10** 835–841

Skudder PJ, Thomas EL, Pavey JA & Perkin AG 1981 Effect of adding potassium iodate to milk before UHT treatment. 1. Reduction in the amount of deposit on the heated surfaces. *Journal of Dairy Research* **48** 99–113

Stapelfeldt H, Olsen CE & Skibsted LH 1999 Spectrofluorometric characterization of β-lactoglobulin B covalently labelled with 2-(4′-maleimidylanilino) naphthalene-6-sulfonate. *Journal of Agricultural and Food Chemistry* **47** 3986–3990

Swaisgood HE 2003 Chemistry of the caseins. In *Advanced Dairy Chemistry Volume 1: Proteins, 3rd Edition*, pp. 139–202 (Eds PF Fox & PLH McSweeney). New York: Kluwer Academic/Plenum Publishers

Tanaka N, Tsurui Y, Kobayashi I & Kunugi S 1996 Modification of the single unpaired sulfhydryl group of beta-lactoglobulin under high pressure and the role of intermolecular S-S exchange in the pressure denaturation. *International Journal of Biological Macromolecules* **19** 63–68

VanCamp J, Messens W, Clement J & Huyghebaert A 1997 Influence of pH and calcium chloride on the high-pressure-induced aggregation of a whey protein concentrate. *Journal of Agricultural and Food Chemistry* **45** 1600–1607

https://doi.org/10.1017/S0022029904000500 Published online by Cambridge University Press