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Functional Improvement of Milk Whey Proteins Induced by High Hydrostatic Pressure

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High pressure is emerging as a new processing technology that produces particular changes in the molecular structure of proteins and thus gives rise to new properties inaccessible via conventional methods of protein modification. This review deals with the main effects of high hydrostatic pressure on the physicochemical characteristics of milk whey proteins and how modifications in their structural properties contribute to functionality. In this paper the mechanism underlying pressure-induced changes in β -lactoglobulin, α -lactalbumin, and bovine serum albumin is explained, and related to functional properties such as gel-forming ability, emulsifying activity, or foaming capacity. The possibility of using high pressures to favor chemical reactions of proteins with other food components, such as carbohydrates, to produce novel molecules with new food uses is also considered.

Keywords high hydrostatic pressure, whey proteins, β -lactoglobulin, functional properties, technological improvements

INTRODUCTION

The use of high pressure has been investigated since the 1980s as an alternative to classical food processing methods. Although the main focus of high pressure technology was initially food preservation, it was recently identified for its potential to improve food texture and produce valuable materials by controlled enzymatic reaction. This is so because pressure treatments specifically influence the functional properties of food biopolymers, mainly proteins, but also polysaccharides and their mixtures. A number of reviews has been published on the effects of high pressure on microorganisms and food components and its applications in the food industry (Farr, 1990; Cuoghi, 1993; Knorr, 1993, 2002; Cheftel, 1995; Tauscher, 1995; Smelt, 1998; Thakur and Nelson, 1998; Tewari et al., 1999; Lillford and Howker, 2000; Mermerlstein, 1998; Meyer et al., 2000; Farkas and Hoover, 2001; San Martín et al., 2002; Sizer et al., 2002; Matser et al., 2004) including pressure-assisted freezing and thawing (Knorr et al., 1998; Cheftel et al., 2000, 2002).

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ABBREVIATION KEY: α -La = α -lactalbumin, β -Lg = β -lactoglobulin; BSA = bovine serum albumin, DS = dextran sulfate; FTIR = Fourier transform infrared spectroscopy; SAXS = small-angle X-ray scattering; VP-FTIR = variable-pressure Fourier transform infrared spectroscopy; WPC = whey protein concentrate; WPI = whey protein isolate.

The basic thermodynamic approach to pressure-induced changes on proteins is based on the compressibility of molecules and on changes of volume during the processes (ΔV) as, unlike temperature, pressure only affects the volume of a system (Balny and Masson, 1993; Gross and Jeanicke, 1994; Hermans and Smeller, 1998; Lullien-Pellerin and Balny, 2002). Thus the application of pressure will cause the equilibrium to shift in favor of the state with the lowest overall volume. Whereas the activity and stability of proteins are influenced by many interactions, the major factors that contribute to ΔV , and hence govern pressure effects, include the electroconstriction of charged and polar groups, elimination of packing defects and the solvation of hydrophobic groups (Boonyaratanakornkit et al., 2002).

The physical modifications of protein structure typically induced by high pressure are disruption and reformation of hydrogen bonds, rupture of hydrophobic interactions and separation of ion pairs. These changes depend on protein structure, pressure, temperature, pH, ionic strength, solvent composition, and protein concentration. Protein denaturation involves dissociation of oligomeric proteins, unfolding, and aggregation. The denaturing mechanism gives rise to intermediate forms, such as the molten globule state, and may eventually lead to multiple denatured forms. During this process, non-covalent forces are cleaved, while covalent bonds are unaffected (Hayakawa et al., 1996). Thus, pressure has a disruptive effect on the quaternary

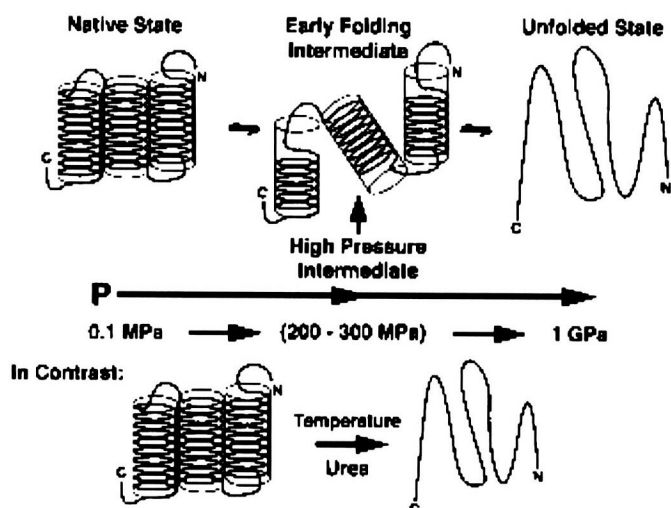


Figure 1 Schematic diagram that compares the use of pressure and temperature in protein denaturation studies. Taken from Jonas (2002) with permission.

(<150 MPa) and tertiary (>200 MPa) structure of most globular proteins but relatively little effect on the secondary structure (approximately >300–700 MPa). According to Jonas (2002), pressure denaturation is more easily controlled and represents a less drastic perturbation of protein structure than thermal of chemical denaturation (Figure 1).

Milk is a source of proteins that fulfill the requirements of being natural ingredients with excellent functionality because of their hydration, interfacial, aggregation, and gelation properties (Wong et al. 1996). Nowadays, separation technologies provide the basis for adding value to milk through the production of proteins that provide the food industry with ingredients to meet specific needs (Huffman and Harper, 1999). Milk whey proteins can be processed using high hydrostatic pressure to ameliorate and expand their uses in the food industry. Obviously, a detailed kinetic and thermodynamic characterization of pressure effects on protein structure is required for the successful application of this technology to the improvement of the functional properties of proteins (Stapelfeldt and Skibsted, 1999).

In this review, the mechanism underlying pressure-induced changes in whey proteins is explained and related to functional properties such as emulsifying activity, foaming capacity or gel-forming ability. The possibility of using high pressures to control enzymatic reactions or to favor chemical reactions, as those between proteins with carbohydrates, to produce novel molecules with new food uses is also considered.

EFFECT OF HIGH PRESSURE ON THE STRUCTURE OF WHEY PROTEINS

The term whey proteins is used to describe the group of milk proteins soluble at pH 4.6 and 20 °C (Farrell et al., 2004). Commercial products termed whey protein isolates (WPI) or concentrates (WPC) are obtained from cheese manufacture at higher pH and will contain intact caseins, as well as their proteolytic products. There is a growing interest in the use of

whey proteins as food ingredients. β -lactoglobulin (β -Lg) is the dominant whey protein and it is largely responsible for the physicochemical characteristics of whey, contributing to solubility, gelation, foaming, emulsification, and flavor binding (Wong et al., 1996). Both the A and B genetic variants occur at high frequency in most breeds of cows and the presence of one or the other of these two variants affects the properties of the milk markedly. β -Lg is a globular protein formed of 162 amino acid residues that contains two disulfide bridges and one free cysteine. It consists predominantly of an eight-stranded antiparallel β -barrel, that forms a binding pocket for small hydrophobic ligands, one short α -helix and a ninth β -strand. β -Lg is categorized as a member of the lipocalin superfamily of proteins (Papiz et al., 1986; Wu et al., 1999; Kontopidis et al., 2004).

Pressure-Induced Unfolding of β -Lactoglobulin

High pressure treatment of β -Lg leads to protein unfolding, as revealed by a reduction in the residual enthalpy of denaturation, characteristic of a partial structure loss (Dumay et al., 1994; Galazka et al., 1996 b). At relatively low pressures, 50 MPa, analysis of thiol reactivity (Møller et al., 1998; Stapelfeldt et al., 1999) and NMR studies (Tanaka and Kunugi, 1996) suggested the existence of a "pre-denatured" state of β -Lg. Such "pre-denatured" form corresponds to a not completely unfolded structure, that precedes reversible denaturation. In a NMR study conducted in our laboratory, we found that the degree of H/D exchange was very small at 100 MPa and there were no variations in the resonances belonging to the strongly bonded "core" of β -Lg (Belloque et al., 2000). These observations suggest that the regions of β -Lg affected by the "pre-denatured" state are likely to be different from the "core," which is still tight at 100 MPa.

Between 100 and 200 MPa, important structural changes occur as evidenced by fluorescence measurements under pressure. The emission maximum red-shifted at pressures above 100 MPa indicating pressure-induced folding changes (Dufour et al., 1994; Stapelfeldt et al., 1996; Tanaka et al., 1996a). According to Stapelfeldt et al. (1996) the intrinsic tryptophan fluorescence increased up to 200 MPa and a further increase of hydrostatic pressure had no effect on the fluorescence quantum yield. However, other authors reported that the fluorescence intensity decreased at higher pressures (Dufour et al., 1994; Tanaka et al., 1996a). These discrepancies could be attributed to the concentration-dependent pressure sensitivity of β -Lg, which aggregates at high concentrations preventing the reformation of the secondary structure, or to the different pressure-time profiles used in the different studies (Dumay et al., 1994; Galazka et al., 1996 b; Stapelfeldt et al., 1996).

On the other hand, some circular dichroism studies on pressurized β -Lg showed no detectable changes in the secondary structure and only a 10% loss of tertiary structure even at 900 MPa (Pittia et al., 1996; Iametti et al., 1997). Nevertheless,

significant modifications in the secondary and tertiary structures of β -Lg following pressurization have been described by other authors (Hayakawa et al., 1996; Tedford et al., 1999a). It should be noticed that, in agreement with variable-pressure Fourier transform infrared spectroscopy (VP-FTIR) studies, most pressure-induced changes on the secondary structure of β -Lg were reversible (Hosseini-nia et al., 1999). Similarly, Møller et al. (1998) found the half-denaturation pressure of β -Lg to be around 140 MPa, but also showed that the exposure of the thiol group occurring at that pressure decreased after 2 days, a result that was attributed to the refolding of the molecule.

Fourier transform infrared spectroscopy (FTIR) and small-angle X-ray scattering (SAXS) revealed that, at approximately 130 MPa, the amount of β -sheet and α -helical structures of β -Lg decreased and the content of disordered structures increased, indicating the beginning of unfolding, but the protein did not unfold completely even at 1000 MPa (Panick et al., 1999). Belloque et al. (2000) reported a considerable H/D exchange of the NH-indole group of Trp¹⁹ and the backbone NHs from several residues belonging to strands FGH of β -Lg at 200 MPa, which was almost complete after treatment at 300 and 400 MPa. This indicates a high flexibility of the entire structure of β -Lg that could be consistent with the existence of an unfolded state at these pressures. However, it was found that the conformation of the β -Lg "core" was identical to the native structure once the pressure had been released, suggesting the formation of a flexible, but still partially structured "core" of β -Lg during pressurization, that may allow the protein to refold back (Belloque et al., 2000).

High-resolution, high-pressure NMR, a novel technique that allows "in situ" experiments, has also been used to investigate the unfolding of β -Lg at pH 2.0 and pressures up to 200 MPa (Kuwata et al., 2001). As illustrated in Figure 2, Kuwata et al. (2001) described that, as pressure increased, the protein starts to show a local conformational fluctuation (excursion of N to N'), leading to two major intermediate unfolded states, with local unfolding either in the hydrophobic core (termed I₂) or in the

opposite side of the barrel (termed I₁). At 200 MPa, a totally unfolded structure (U) becomes dominant.

Pressure-Induced Aggregation of β -Lactoglobulin

Pressure-induced unfolding appears to increase surface hydrophobicity of β -Lg, through the unmasking of buried hydrophobic groups, leading to protein aggregation. Such changes are partially reversible and it may be difficult to discern whether they mainly take place under pressure or after pressure release (Dumay et al., 1994; Galazka et al., 1996b; Pittia et al., 1996; Hosseini-nia et al., 1999). It has been hypothesized that the situation is similar to what occurs during thermal denaturation of β -Lg. Thus, reversible structural modifications lead to transient dimer dissociation, mainly by destabilization of hydrophobic interactions and, above a threshold of treatment intensity, monomers unfold and interact to give aggregates that are stabilized by covalent and non-covalent interactions (Iametti et al., 1997; Panick et al., 1999). Stapelfeldt and Skibsted (1999) included an additional initial step without a parallel in thermal denaturation that involves the moderate pressure (up to 50 MPa) induced changes in β -Lg, described as pressure melting, that enhance thiol reactivity.

Evidence for the contribution of disulfide bonds to polymerization has been provided (Galazka et al., 1996b; Tanaka et al., 1996c; Iametti et al., 1997). Studies conducted during storage of pressurized β -Lg solutions showed that the formation of intermolecular disulfide bonds continues after pressure release (Funtenberger et al., 1995). Disulfide bonded aggregates have been reported to form by SH/SS exchange reactions, rather than by SH oxidation, with the participation of the free -SH of Cys¹²¹, buried in the native protein (Funtenberger et al., 1997; Tanaka et al., 1996c). According to Belloque et al. (2000), intermolecular disulfide bonds, are likely to involve Cys⁶⁶-Cys¹⁶⁰, rather than Cys¹⁰⁶-Cys¹¹⁹, because the resonances of residues such as Cys¹⁰⁶ and Met¹⁰⁷, located in and around the Cys¹⁰⁶-Cys¹¹⁹ disulfide bond, did not change after pressurization in any of the variants. The formation of oligomers linked in such a way may explain the decreased availability of parts of the molecule lying close to the Cys⁶⁶-Cys¹⁶⁰ disulfide bond to enzymatic cleavage after pressure treatment (Knudsen et al., 2002).

NMR spectral data suggested that pressure-induced disulfide bond polymers of β -Lg were made up of structured proteins, rather than compact aggregates (Belloque et al., 2000). In fact, FTIR showed that intermolecular β -sheet formation is of less importance in pressure-induced than in thermally-induced aggregation of β -Lg, what probably points to the absence of extensive hydrophobic interactions during the aggregation process (Panick et al., 1999).

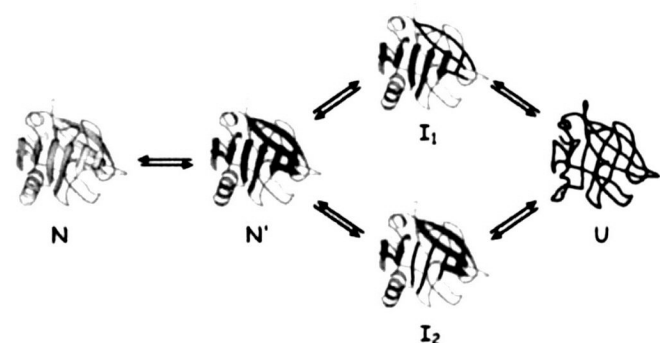


Figure 2 Conformational equilibrium of β -lactoglobulin revealed by high pressure NMR. N, native state; N', the low-lying excited state of N; I₁, intermediate conformer with disordered "non core" region (β B-E); I₂, intermediate conformer with disordered "core region" (β F-H); U, fully unfolded conformer. Taken from Kuwata et al. (2001) with permission.

Pressure-Induced β -Lactoglobulin Molten Globule

The molten globular state of proteins is a thermodynamically stable state, intermediate between the ordered and fully unfolded

states. As a molten globule, a protein usually retains secondary structure and little specific tertiary structure, but with increased mobility and looser packing (Qi et al., 2001). According to Yang et al. (2001), pressurization at 600 MPa and 50°C induces β -Lg to form a stable molten globule. Such a structure, stabilized as a disulfide linked dimer, remained unchanged for at least 3 months and may display enhanced functionality for use as a food ingredient (Yang et al., 2001). The molten globule state induced by high pressure was not found to exhibit increasing affinity for hydrophobic flavor compounds or ligands (Yang et al., 2003). In fact, conformational changes in the calyx decreased retinol binding to molten globular β -Lg. Nevertheless, it was shown that the blocking of the free thiol group during the high pressure treatment retained or increased this ability and may produce modified β -Lg to be used as a retinol carrier for acid foods (Yang et al., 2002).

Influence of pH, Protein Concentration and Temperature on Pressure-Induced β -Lactoglobulin Denaturation

Stability of proteins under pressure largely depends on pH. Fluorescence measurements under pressure revealed that β -Lg unfolding was more extensive and irreversible at pH 7.0 than at pH ≤ 3.0 (Dufour et al., 1994; Ikeuchi et al., 2001). At pH 5.6, because the pH approaches the isoelectric point (5.2), the protein has some positive and some negative charges and electrostatic interactions tend to favor the folded state (Tedford et al., 1999b). It should be noticed that the reactivity of the SH groups is enhanced at neutral and alkaline pH (Funtenberger et al., 1997; Famelart et al., 1998), and this contributes to the irreversibility of unfolding (Ikeuchi et al., 2001). Furthermore, some of the effects of high pressure on protein structure could also indirectly derive from pH changes, or changes in the dielectric constant of the medium, due to pressure. Under high pressure, the equilibrium $\text{CH}_3\text{COOH} \leftrightarrow \text{CH}_3\text{COO}^- + \text{H}^+$ favors the ionic species and causes a drop of, approximately 0.2–0.3 units per 100 MPa of pressure applied (Kitamura and Itoh, 1987; Heremans, 1995). Therefore, since formation of β -Lg oligomers is promoted at neutral over acidic pHs, extensive polymerization was observed in pressure resistant buffers at pH 7.0, as compared to water or phosphate buffers (Funtenberger et al., 1995). The presence of other compounds can vary the denaturation behavior. For instance, polyols, exert a baroprotective effect on β -Lg, because they favor the hydration of the protein in aqueous solution (Dumay et al., 1994). Any modification of the water shell around proteins will alter the spatial distribution of charges, and this could play a significant role in the evolution of protein conformation under high pressure (Dufour et al., 1994).

Other factors are relevant to the nature of the pressure-induced modifications. Irreversible structural changes and aggregations are enhanced at increasing protein concentrations (Galazka et al., 1996b). On the other hand, there is a dependence of irreversible structural modifications of β -Lg on the duration of the treatment, but only in a limited pressure range (Iametti et al., 1997).

Although there are very few studies on the effects of high pressure at moderate temperatures on protein structure, it has been reported that the pressurization temperature strongly promotes irreversible disruption of β -Lg at the tertiary level (Tedford et al., 1999b; Tedford and Schaschke, 1999; Bull and Schaschke, 2002). The combined use of pressure and temperature has less effects on the secondary structure of the protein and on the temperature of denaturation (Tedford and Schaschke, 2000). Thus, a complete loss of tertiary structure was observed using circular dichroism and DSC following treatments at 100 MPa and 75°C at pH 7.0, and a 72% loss at pH 5.6 (Tedford and Schaschke, 2000). Low temperatures minimize the loss of native structure induced by pressurization and reduce subsequent aggregation reactions. Accordingly, pressure-induced unfolding of β -Lg was significantly lower after processing at 300 MPa and 4 or -2°C than at 25°C (Kolakowski et al., 2001). This is probably due to the existence of a more hydrated state of the protein under high pressure and low temperature conditions as compared to ambient temperature (Kolakowski et al., 2001).

Effect of High Pressure on the Susceptibility of β -Lactoglobulin to Enzymatic Reactions

The effects of high pressure on proteins could be of practical use in controlling certain enzyme reactions. Pressure may induce changes in the conformation of the substrate, which may make the enzymatic attack easier or more difficult, or in the conformation of the enzyme, which may have consequences for the activity. In addition, the activation volume of the reaction will determine the effects of pressure on the catalytic process (Heremans, 1995).

β -Lg has been reported to exhibit an improved proteolytic digestibility when hydrolyzed under high hydrostatic pressure. According to some authors, the enhancement of proteolysis under high pressure conditions is due to conformational changes of β -Lg that make it more susceptible to hydrolysis, rather than on pressure effects on the enzyme or a direct effect on the β -Lg–enzyme interaction (Dufour et al., 1995; Van Willige and Fitzgerald, 1995; Stapelfeldt et al., 1996; Bonomi et al., 2003). In any case, protease activity under pressure is also determined by the enzyme stability, with many monomeric enzymes retaining their native structures up to pressures from 400 to 800 MPa (Kudryashova et al., 1998). Furthermore, the activity of some enzymes, such as thermolysin and chymotrypsin, is greatly increased at 200 and 360 MPa respectively (Mozhaev et al., 1996; Kunugi et al., 1997). Thus, hydrolysis of β -Lg by thermolysin under pressure at pH 7.0 might be favored by partial unfolding of the substrate and pressure-induced thermolysin activation, whereas enhanced hydrolysis by pepsin at its optimum pH (2.5–3.0) occurred without pressure-induced differences in the cleavage sites (Dufour et al., 1995). Negative reaction volume changes were probably the major factor favoring peptic hydrolysis of β -Lg under pressure, since this protein seems to resist

unfolding by pressures up to 300 MPa applied at 25°C and acidic pH (Dufour et al., 1995).

According to Maynard et al., (1998) tryptic hydrolysis of β -Lg at pH 7.7 was accelerated under pressure, with an optimum at 300 MPa, although these authors reported that the nature of the tryptic end-products was the same than those obtained at 0.1 MPa. Steady-state kinetic experiments showed that such enhanced digestibility results from an increase in the catalytic constant up to 100 MPa, with pressure presumably promoting exposure of normally hindered cleavage sites, and from a continuous decrease in Michaelis constant, which indicates changes in the protein conformation that facilitate binding of the substrate to the enzyme (Olsen et al., 2003).

On the other hand, since pressure-induced denaturation of β -Lg is slowly reversed following pressure release, pressurization also enhances the susceptibility of the protein to subsequent proteolysis at atmospheric pressure (Stapelfeldt et al., 1996; Otte et al., 1997; Maynard et al., 1998; Knudsen et al., 2002; Bonomi et al., 2003). Differences were noticed between the peptide profiles resulting from hydrolysis of pressure-treated and unpressurized β -Lg (Knudsen et al., 2002).

In a different approach, high pressure treatment of β -Lg in the presence of transglutaminase was used to induce enzymatic crosslinking. Microbial transglutaminase, an enzyme capable of forming inter- and intramolecular bonds between protein-bond Gln and Lys residues, remains active even after incubation for 2 h at 400–600 MPa and 40°C (Lauber et al., 2001a). β -Lg (1% solution) is not affected by the enzyme at atmospheric pressure, but it was oligomerized under or after high pressure treatments (400 MPa, 40°C, 60 and 120 min, respectively), although in the latter case, crosslinking was lower (Lauber et al., 2001 b). High pressure treatments of solutions of β -Lg and β -casein led to the formation of heterologous oligomers of both proteins, probably due to the increase in the hydrophobic groups on β -Lg, what results in a closer approach of the protein to the active site of the enzyme (Lauber et al., 2003).

Effect of Pressure on the Denaturation of β -Lactoglobulin Variants A and B

Although Panick et al., (1999) did not find differences in the pressure stability of the most common genetic variants of β -Lg, A and B, by FTIR and SAXS, other workers have reported variations in their denaturation response towards pressure. The β -Lg variant A denatures faster by heat what probably arises from the structural differences caused by the changes of the residue 64 (Asp in β -Lg A and Gly in β -Lg B) and 118 (Val in β -Lg A and Ala in β -Lg B) (Boye et al., 1997). VP-FTIR spectra showed a different conformational behavior upon compression and decompression of β -Lg A, B, and AB, and revealed that the structure of β -Lg A was more pressure sensitive than that of β -Lg B, while β -Lg AB underwent less aggregate formation than β -Lg A (Hosseini-nia et al., 1999). In fact, the deuteration of the backbone NH of Met¹⁰⁷, followed by 1D ¹H NMR, showed that the "core" of β -Lg A became

more flexible than that of β -Lg B after pressurization at 200 MPa for 5 min, although refolding was equally complete for both variants (Belloque et al., 2000). This suggests that the functional or biological attributes of the different genetic variants of β -Lg might be differentially affected by high hydrostatic pressure (Hosseini-nia et al., 1999). However, Van Willige and Fitzgerald (1995) reported similar rates of enzymatic hydrolysis of β -Lg A and B at 100 and 300 MPa, despite the fact that β -Lg A is hydrolyzed faster than β -Lg B at atmospheric pressure.

Pressure-Induced Denaturation of α -Lactalbumin

α -lactalbumin (α -La) is a compact globular calcium-binding protein that makes approximately 3% of the total milk protein and 13% of the total whey protein. The molecule contains 123 amino acids and has four disulfide bonds (Farrell et al., 2004).

Hinrichs et al. (1996) determined orders of reaction of $n = 2.0$ for α -lactalbumin (α -La) and $n = 2.5$ for β -Lg in a whey protein isolate (WPI). These reaction rate constants were found to vary slightly at higher protein concentrations (Keim and Hinrichs, 2004). Comparison between pressure-induced structural changes in α -La and β -Lg confirmed a higher stability of α -La. Reversible unfolding of α -La started at 200 MPa and loss of native structure was irreversible at 400 MPa, as compared with 50 and 150 MPa, respectively, for β -Lg. (Tanaka and Kunugi, 1996; Tanaka et al., 1996a and b). α -La is present in a molten globule state beyond 200 MPa up to 400 MPa (Jonas, 2002).

The different behavior of both proteins was explained by the differences in their secondary structures, that lead to a higher effective hydrophobicity in β -Lg, and/or in the number of disulfide bonds (four in α -La and two in β -Lg) and Ca²⁺ binding sites (Tanaka and Kunugi, 1996). In fact, the binding of calcium remarkably stabilizes α -La against pressure by a 200 MPa increase of the value of pressure at which denaturation occurs (Dzwolak et al., 1999; Hosseini-nia et al., 2002). Pressure-induced oligomerization of α -La alone was not observed in the absence of low molecular weight thiol reducers, even at high pressures applied for extended periods (Jegouic et al., 1996). In mixtures of α -La and β -Lg, and due to its free -SH group, β -Lg can trigger the oligomerization of α -La resulting in the formation of a large heterogeneous population of oligomers, presumably composed of several hundreds of β -Lg and α -La molecules (Jegouic et al., 1997; Grinberg and Haertlé, 2000).

Pressure-Induced Denaturation of Bovine Serum Albumin

The properties of other milk proteins, such as bovine serum albumin (BSA), are also modified by pressure (Hayakawa et al., 1992). BSA is a globular protein that accounts for approximately 8–10% of the proteins of whey. As found in milk,

this protein is physically and immunologically identical to blood serum albumin. The complete amino acid sequence of BSA contains 583 aminoacids and, of the 35 Cys residues per molecule, 34 per monomer form 17 disulfide bonds (Farrell et al., 2004).

Unlike the case of β -Lg, processing at 800 MPa was reported to have a substantial effect on the secondary structure of BSA (Galazka et al., 1996b; Galazka et al., 1997). However, pressure-induced changes in the secondary structure of BSA were mainly reversible (Hosseini-nia et al., 2002). The relatively high number of disulfide linkages in BSA may impede pressure-induced aggregation by protecting the hydrophobic groups present inside the molecule from exposure to the solvent (Hosseini-nia et al., 2002). Nevertheless, at higher pressures BSA can also polymerize through disulfide bonding involving the free -SH residue (Galazka et al., 1996b; Galazka et al., 1997).

USE OF HIGH PRESSURE TO MODIFY THE FUNCTIONAL PROPERTIES OF WHEY PROTEINS

As already mentioned, pressure alters the balance of intramolecular, intermolecular, and solvent-protein interactions with a tendency to increase the area of protein molecules accessible to the solvent. Therefore, like many other physical and chemical procedures that alter protein structure, high pressure can be used to modify functional properties, such as gelling, emulsifying, whipping, and dough-forming properties of food proteins (Messens et al., 1997; Galazka et al., 2000c; Dumolin and Hayashi, 1998). β -Lg, being the major whey protein, largely determines the functional behavior of whey protein products under pressure, in terms of emulsifying properties (Galazka et al., 1995), foamability (İbanoğlu and Karatas, 2001), or gel forming ability (Dumay et al., 1998; Van Camp and Huyghebaert, 1995a). In addition, β -Lg is a major allergen in cow's milk and enzymatic hydrolysis under high hydrostatic pressure has been proposed as a way to produce hypoallergenic hydrolysates (Bonomi et al., 2003).

Interfacial Properties

Pressurized (300-900 MPa) β -Lg displays lower emulsifying and foaming capacity than native β -Lg, probably because structural changes, leading to an increased hydrophobicity and aggregate formation, reduce both the diffusibility and the concentration of protein available for adsorption to interfaces (Pittia et al., 1996). Other food proteins also form emulsions with higher droplet size and faster creaming after pressurization (Galazka et al., 1999a). However, it should be noticed that the higher surface hydrophobicity of pressure treated β -Lg is responsible for a greater capacity for protein-protein interactions in the adsorbed layers of interfaces (Pittia et al., 1996). Thus, native β -Lg forms highly viscous films that increase in viscosity on pressurization, whereas BSA forms less viscous

films that weaken after high pressure treatment (600 MPa), as a result of the decreased surface hydrophobicity, unfolding, and aggregation of the pressurized BSA (Galazka et al., 2000b).

Treatment of β -Lg up to 800 MPa led to a loss of emulsifying capacity and a reduction in the stability of fine emulsions made with 0.4% (w/w) β -Lg and 20% (v/v) oil (Galazka et al., 1996a). However, the stability of emulsions formed with the same concentration of native protein changed only slightly on pressurization. This suggests that the structure of adsorbed β -Lg at the surface of the droplets is much less affected by the treatment, since the molecules are already partially unfolded and interacting strongly with neighboring molecules in the interfacial layer (Galazka et al., 1996a; Dickinson and James, 1998). The emulsifying behavior of WPC (0.4%, w/w protein, 20%, v/v *n*-tetradecane, pH 7), pressurized before or after homogenization, was similar to that of β -Lg (Galazka et al., 1995).

High pressure treatments (>400 MPa) of emulsions stabilized with a higher concentration of β -Lg (1% w/w protein and 40% v/v oil), led to an extensive increase in viscosity, with the generation of gel-like characteristics (Dickinson and James, 1998). The proportion of free protein present in the aqueous continuous phase greatly influenced the degree of emulsion flocculation following pressurization, as unfolded proteins aggregate in solution and bind adsorbed proteins at the surface of oil droplets (Dickinson and James, 1998).

The extent of the pressure-induced changes in emulsion properties is determined by the length and severity of the treatment conditions (Dickinson and James, 1999). Pressurization of emulsions stabilized with β -Lg at 450 MPa at 25°C increased the viscosity over those processed at 10°C, while gelled emulsions were formed upon treatment at 40°C (Dumay et al., 1996). Furthermore, viscoelasticity of emulsion gel samples increased on pressurization with increasing ionic strength and at pH values close to the isoelectric point, that is, under conditions favoring a loss of electrostatic stability (Dickinson and James, 1998). In addition, it is possible to control the extent of pressure-induced flocculation in β -Lg emulsions by displacing this protein from the oil-water interface by sodium caseinate and Tween 20, as this reduces its availability for cross-linking among surfaces (Dickinson and James, 1999). Nevertheless, intense high pressure treatments (800 MPa, 60 min) were found equivalent to relatively mild thermal treatments (65°C, 5 min) in destabilizing these emulsion systems (Dickinson and James, 1998).

There is some evidence that pressure-treated β -Lg has improved foaming properties even though it gives emulsions with larger droplets and lower stability. This discrepancy may be explained in terms of the different degrees of aggregation undergone by the protein in the two cases, since a higher concentration of protein was used for the emulsions (Galazka et al., 2000b). Pressurization of whey protein isolate (WPI) at high concentration (2% w/v), or in high buffer molarity, diminished its foaming properties (İbanoğlu and Karataş, 2001).

Gelling Properties

Whey proteins can form gels under pressure whose strength normally increases with the pressure level and the duration of the treatment (Van Camp and Huyghebaert, 1995a; Famelart et al., 1998; Kanno et al., 1998; Fertsch et al., 2003). Van Camp and Huyghebaert (1995a) studied the effect of pH on gel formation from whey protein concentrates (WPC) (140 g/l of protein) and found maximum gel strength in phosphate buffer at pH 8.0–9.0 and in water at pH 7.0–9.0. Similarly, Famelart et al., (1998) reported that WPC (97–127 g/kg protein) gelled at pH 9.0 and pressures from 200 to 400 MPa. No gel formation occurred at acidic pH, except in the presence of NaCl, nor after the addition of a –SH blocking agent. This confirmed the role of –SH groups in the gelation process (Van Camp and Huyghebaert, 1995a, Van Camp et al., 1997). Therefore, above the isoelectric point, disulfide bonds dominate and hydrophobic interactions appear to contribute very little to pressure-induced whey protein gels, while at acidic pH, whey protein gels were mainly driven by electrostatic interactions and, possibly, also hydrogen bonds (Van Camp et al., 1997). The amount of disulfide bonds, quantified by means of an extraction test, was found responsible for gel strengthening, firmness, and elasticity of pressure-induced whey protein gels (Keim and Hinrichs, 2004). A protein concentration increase was also related to an increase in gel strength (Van Camp and Huyghebaert, 1995a, Kanno et al., 1998). Nevertheless, in some studies a plateau region in gel strength was reached, meaning that over a certain concentration no more interactions occurred as the protein content was enhanced (Van Camp and Huyghebaert, 1995a; Zasytkin et al., 1996; Famelart et al., 1998).

Pressure-induced gels are generally considered to retain better flavor and color and to exhibit softer texture than heat-induced gels (Okamoto et al., 1990). Heat-set gels of WPC or β -Lg isolate are firmer and more elastic than pressure-induced gels of the same protein concentration (Van Camp and Huyghebaert, 1995a and b; Zasytkin et al., 1996). Electron microscopy of pressure-induced gels showed a porous structure maintained with few, weak, and short-term intermolecular cross-links as compared with heat-induced gels (Van Camp and Huyghebaert, 1995b). In contrast to the continuous, finely stranded aggregates with high water retention of heat-induced gels (87°C, 45 min), pressure-induced gels (450 MPa, 15–30 min at 25°C) of β -Lg (9–15% w/w in water, pH 7.0), displayed porous microstructures prone to exudation. An increase in the initial protein concentration (up to 15%) induced even larger pores and pillars, leading to protein microparticulation and local microphase separation (Dumay et al., 1999; Zasytkin et al., 1996). Moreover, pressure-induced gels of β -Lg could be totally solubilized in water after pressure release, stressing that only weak interactions contributed to gel formation. However, a marked strengthening of the network occurred on storage at 4°C as a result of a time-dependent formation of hydrophobic interactions and disulfide bonds (Dumay et al., 1998).

It was concluded that pressure promoted incomplete gelation, due to partial unfolding and random aggregation of β -Lg, as compared to heating, reducing the ability of the protein network to immobilize water (Dumay et al., 1999). As already mentioned, when explaining the pressure-induced aggregation of β -Lg, if intermolecular associations in pressure-induced gels are driven mainly by the formation of disulfide bonds at an edge of the protein, with little hydrophobic interaction, this would provide β -Lg units with more freedom of movement than in the case of hydrophobic bound aggregates, reducing the rigidity of the gel (Belloque et al., 2000). At 15% β -Lg concentration, aggregation predominated over unfolding, leading to protein precipitation as microparticles (Dumay et al., 1999).

By increasing the pressurization temperature it is possible to increase the strength of the whey protein gels formed and decrease the pressure and treatment time needed for the same gel strength (Van Camp et al., 1996). Although the pressure-temperature dependency of gelation varies with the protein system, it should be noticed that harder gels were also formed at low temperatures and moderate pressures in the case of other proteins, such as ovalbumin (Dumolin et al., 1998). It was also attempted to produce heat-induced gels from pressurized proteins. Solutions of β -Lg (5%) were pressurized (450 MPa) and either heated immediately or after 24 h to induce thermal gelation. Protein aggregation increased over storage time, leading to a higher proportion of polymers and this negatively influenced gel strength after heat treatment (Olsen et al., 1999).

Hydrostatic pressure treatment (100–400 MPa, 30 min, 20°C) of α -La samples (10%, w/w in water pH 6.96) did not change the magnitude of rheological parameters and there was no gel formation (Ahmed and Ramaswamy, 2003). However, WPC (15–20% w/w) formed stronger gels than an equivalent concentration of β -Lg (7.3–9.7% w/w) on pressurization (300 MPa, 30 min), and this effect was assigned to the incorporation of α -La in the network (Ipsen et al., 2002).

Walkenström and Heremansson (1996, 1997a and b) combined temperature and high pressure to form mixed gels of gelatin (a cold-set protein of pI ≥ 7.5) and whey proteins with different microstructures and morphologies. The WPC network set first when temperature was used for gel formation, giving phase-separated, bicontinuous networks, which followed the properties of either of the pure components, depending on the ratio between the polymers (Walkenström and Heremansson, 1996). When high pressure was used, a gelatin network set first, forming a gelatin continuous system with spherical inclusions of WPC at pH 5.4 or a dense network in which neither gelatin nor the WPC network could be identified (Walkenström and Heremansson, 1997a). By cooling under high pressure, both networks could be formed simultaneously, leading to different microstructures depending on the pH: a gelatin continuous system at pH 5.4 and a complex coacervate at pH 7.5 (Walkenström and Heremansson, 1997a).

Production of Hypoallergenic Hydrolysates

The enhanced proteolytic digestibility of β -Lg under pressures of 150–200 MPa has been applied to its selective removal from whey concentrates to be used as hypoallergenic additives to modified milk for infants (Hayashi et al., 1987; Okamoto et al., 1991). A low immunochemical reactivity was reported for tryptic and chymotryptic hydrolysates of β -Lg produced during pressure treatments at 600 MPa for 10 min (Bonomi et al., 2003). Furthermore, a reduced antigenicity was reported after hydrolysis at atmospheric pressure of pressurized β -Lg (Nakamura et al., 1993). According to Bonomi et al. (2003), transient conformers of β -Lg form under conditions of subdenaturing pressure intensity. These present ample regions of the hydrophobic core unfolded and thus accessible to enzymatic hydrolysis, what might eventually lead to the removal of epitopes.

EFFECT OF PRESSURE ON THE INTERACTIONS BETWEEN MILK PROTEINS AND CARBOHYDRATES FUNCTIONAL APPLICATIONS

Effect of Pressure on Maillard Reaction

Several studies have evaluated the effects of pressure on chemical reactions of proteins with other food components, such as carbohydrates. High pressure and, in particular, its combination with moderate temperatures, may influence Maillard reaction with important consequences to the flavor, color, and nutritional value of foods. The measurement of volumes of activation for products of model Maillard reactions indicated that pressure could accelerate the initial condensation processes, but slow down the formation of final heterocyclic products, melanoidins, and volatiles, because the decomposition of the Amadori rearrangement product has a positive volume of activation (Tamaoka et al., 1991; Isaacs and Coulson, 1996; Bristow and Isaacs, 1999). Further work confirmed that high pressure affected in different ways the different stages of Maillard reaction and that such effects were strongly influenced by the pH of the systems (Hill et al., 1996, 1998, 1999; Moreno et al., 2003).

Although more research is required to understand the effects of pressure on Maillard reaction, especially in view of the complexity and number of processes involved, results obtained so far suggest that high pressures could be used for its control. This would be of especial interest when Maillard reaction is deliberately pursued, as an alternative to chemical means, to produce protein-carbohydrate conjugates as new ingredients with improved functional characteristics. Such biopolymers can act as ideal steric stabilizers as they join the emulsifying and foaming role of the protein and the water-holding role of the carbohydrate (Haertlé and Chobert, 1999). This approach has not as yet been applied to the production of glycosylated whey proteins but, for example, a bifunctional lysozyme-dextran complex with excellent antimicrobial and emulsifying activities was prepared by Maillard reaction in the liquid state at 192.14 MPa. The use of

high pressure shortened the reaction period and suppressed the formation of melanoidins, although it decreased the production rate of the complex (Nakamura et al., 2001).

Effect of Pressure on Non-Covalent Interactions between Proteins and Carbohydrates

In addition to covalent coupling, such as Maillard-type linkage, electrostatic interactions can also be used to bring together proteins and carbohydrates, avoiding the use of undesirable chemicals and yielding emulsifiers with good solubility and emulsion stabilizing properties (Dickinson and Galazka, 1991). Below their isoelectric point, proteins can establish strong electrostatic associations with anionic polysaccharides. At neutral pH, both usually carry a negative net charge, but electrostatic interaction may still take place between the negative charges on polysaccharides and positively charged patches on proteins. Such interactions, reinforced with non-ionic attractions, lead to complex formation (Dickinson, 1998). High pressure influences both protein conformation and electrostatic interactions, therefore non-covalent protein-polysaccharide interactions are also susceptible to considerable modification (Dickinson and James, 2000).

There are several examples in the literature on the use of high pressure to promote non covalent associations between milk proteins, mainly β -Lg or BSA, and anionic polysaccharides. The pressurization of BSA in the presence of dextran sulfate (DS), an anionic polysaccharide that forms electrostatic complexes with BSA at pH 7.0 and low ionic strength, was shown to reduce the degree of unfolding of the protein at 800 MPa (Galazka et al., 1996b). Furthermore, DS exerted a protective effect against BSA aggregation caused by disulfide bonding (Galazka et al., 1997). The application of pressure was found to induce the formation of stronger complexes between these biopolymers. Similar results were obtained when mixtures of BSA and other sulfated polysaccharides, such as ι - or κ -carrageenan, were subjected to pressures of 600 MPa (Galazka et al., 1999b). It was hypothesized that, in view of the disruptive effect of pressure on ionic bonds, the electrostatic protein-polysaccharide complex could dissociate under pressure and rapidly reassociate on pressure release, the strength of the interaction being related to the density of sulfate groups on the polysaccharide. Since under pressure the protein may expose more charged groups, electrostatic BSA-polysaccharide interactions could be restored more strongly than before, protecting BSA against protein-protein aggregation and the consequent loss of functionality (Galazka et al., 1997, 1999b, 2000b). The possible mechanism, involving complex dissociation under pressure, is illustrated in Figure 3 (Galazka et al., 1999b).

Because high pressure influences the nature of protein-polysaccharide interactions, it has the potential of controlling the viscoelasticity and stability of emulsions containing these macromolecules. In fact, pretreatment of BSA (400–700 MPa, pH 7.0) before emulsification affected the rheology of the resulting BSA stabilized emulsions after DS addition

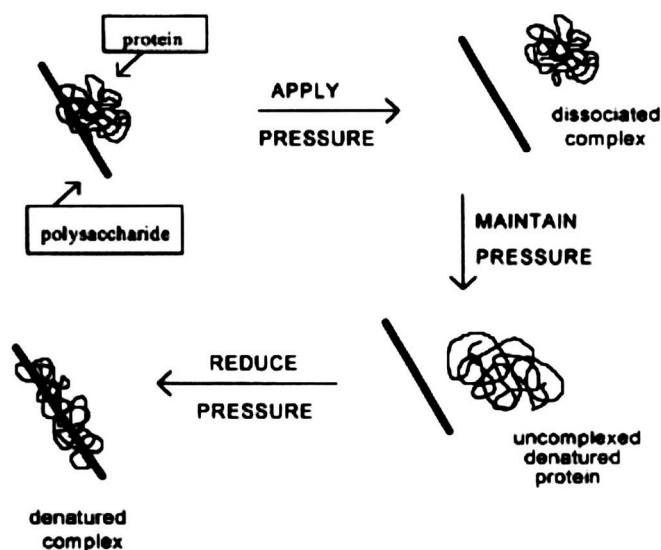


Figure 3 Schematic representation of the effect of high pressure processing on electrostatic protein-polysaccharide complex. Application of pressure leads to the dissociation of the biopolymers, the globular protein unfolds and exposes more charged groups during maintenance of pressure, and the complex of pressure-unfolded BSA with DS reforms during pressure release. Taken from Galazka et al. (1999b) with permission.

by increasing flocculation and associated viscosity (Dickinson and Pawlowsky, 1996). Other studies have dealt with the behavior of similar systems when high pressure was applied to a mixed solution of protein-polysaccharide prior to emulsification or when pressure was applied directly to the emulsion, either before or after the addition of the polysaccharide. However, in many of these studies, food proteins other than milk proteins were used. For instance, emulsions made with pressurized (600 MPa, 20 min) mixtures of ovalbumin and DS had the best emulsifying efficiency and stability, as compared with the untreated or heat-treated (80°C, 10 min) protein or protein-polysaccharide mixtures (Galazka et al., 2000a). Similar results were obtained with high pressure treated mixtures of IIS *Vicia faba* and ι -carragenan or κ -carragenan (Galazka et al., 1999a). However, treatment at 600 MPa of emulsions prepared with mixtures of ovalbumin and DS induced significant levels of flocculation (Galazka et al., 2000a).

On the other hand, the foaming behavior of a solution containing β -Lg (0.15% w/w) and DS at pH 7 was studied after pressurization up to 800 MPa. Improved foaming ability and reduced foam stability were reported. These were attributed to partial denaturation of the protein without aggregation upon pressure release (İbanoğlu, 2001).

Some carbohydrates, such as pectin, possess stabilizing and thickening properties that are commonly exploited in the manufacture of dairy products. Emulsions prepared with pressure-treated β -Lg in the presence of even low quantities of pectin exhibited extensive bridging flocculation, particularly at pH $\leq$ pI (Dickinson and James, 2000). This was attributed to attractive intermolecular interactions between both biopolymers promoted by high pressure that caused interdroplet macromolecular

linkages upon emulsion formation. Significant levels of flocculation at neutral pH also confirmed complexation under conditions where both macromolecules carry net negative charges. The addition of small quantities of low-methoxyl pectin (0.1–0.5%, w/w) enhanced the strength of pressure-induced β -Lg (4–8%, w/w) gels (Dickinson and James, 2000).

Gelation of mixed aqueous solutions of polysaccharides and proteins opens a wider range of mechanical and textural properties as compared to the pure biopolymers. Furthermore, the characteristics of these protein/polysaccharide gels can be effectively modified by the use of high pressure. The addition of sucrose to β -Lg solutions led, on pressurization, to gels with smaller pore size and pillar thickness because of its baroprotective effect against β -Lg unfolding and aggregation (Dumay et al., 1994, 1998). Similarly, pressure-induced gels of β -Lg/xanthan solutions presented smaller pillars and pores and were less prone to exudation than those made from β -Lg alone (Zasyepkin et al., 1996).

The resulting gel texture can be controlled not only by the mode of gelation (temperature or pressure) but also by the polysaccharide concentration and the intensity of processing (Dumay et al., 1999). Mixed β -Lg/anionic polysaccharide solutions (12% w/w protein, 0.1–1% w/w polysaccharide, pH 7.0) were compatible at ambient temperature and atmospheric pressure, but gelled on the application of 450 MPa for 30 min at 25°C. Gels formed at the lowest concentration of polysaccharide exhibited no exudation, as compared with the marked exudation observed in pressure-induced gels of β -Lg alone (Dumay et al., 1999). It was suggested that pressure promotes phase separation as β -Lg partially unfolds and aggregates, leading to a kind of water in water emulsion, with two phases containing different respective concentrations of protein and polysaccharide. As already mentioned, biopolymer electrostatic complexation is inhibited under high pressure, whereas protein and polysaccharide self associations are enhanced. When pressure is released, electrostatic and hydrophobic interactions are restored, but at least one of the two polymers has incurred in irreversible conformational changes (Dumay et al., 1999). For greater polysaccharide additions, gel exudation occurred, depending on the type and concentration of the added polysaccharide, as phase separation seemed to be enhanced for a higher number of net negative charges. It was explained that, under those conditions, the polysaccharide probably acted by promoting protein self-association and increasing β -Lg microparticulation. According to Michel et al. (2001), high pressure offers more versatility than heat to modify the denaturation degree of proteins and to achieve subsequent phase separation. This opens many possibilities to create and control new food textures in mixtures of milk proteins and polysaccharides.

CONCLUDING REMARKS

The effectiveness of high hydrostatic pressure to preserve food has been recognized for long. However, experimental

research on high pressure treatments to modify protein conformation, so as to tailor milk proteins to exert different functions, is more recent. In fact, to date, food industrial applications of high pressure are mainly oriented to safety and stability improvements, while the use of high pressure treatments to induce structural changes that improve protein functionality has scarcely been exploited.

A deeper understanding of the effects of the process is still needed to open new perspectives in the control of the technological properties of proteins, as well as to optimize processing conditions. In particular pressure-temperature behavior of milk proteins and enzymes, as well as reversibility of changes need careful attention. This, together with the development in high pressure equipment for industrial operations would make it feasible to establish cost-effective milk protein processing plants for interesting applications, such as the production of a new range of hypoallergenic and high added-value dairy based products.

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