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Current ways to modify the structure of whey proteins for specific functionalities—a review

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Abstract The native whey proteins have been intensively used in a multitude of food applications due to their high nutritional, biological, and versatile technofunctional properties. The range of applications of whey proteins has further been extended in the last decades by the use of whey protein aggregates offering new technofunctional properties. These properties are directly dependent on the structure of whey protein aggregates, i.e., their size, shape, density, surface properties, and the type of bonds maintaining proteins together in the aggregates. In this review, after a brief description of the major whey proteins, we examine the most important advances reported to date pertaining to the available approaches to modify the structure of whey proteins for specific functionalities. Our laboratory, Science and Technology of Milk and Eggs, has contributed significantly to the advancement of knowledge on the structure-function relationships of whey proteins either in the native or denatured/aggregated forms. Our expertise and research approaches are highlighted throughout some selected results accumulated during the last decade in comparison with results from the literature.

Keywords Whey proteins · Structure · Functionalities · Assemblies

1 Introduction

Milk proteins are classified into two groups (caseins and whey proteins) based on their solubility at pH 4.6 after acidification of a raw skim milk (Farrell et al. 2004). Milk

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proteins in their native form exert highly valuable properties giving them incomparable assets in numerous food applications. In terms of nutrition, milk proteins have well-balanced amino acid composition and the amino acid sequence in the polypeptide chains forms various types of bioactive peptides that are discretely released upon protein hydrolysis (Korhonen and Pihlanto 2006). They are carriers of micronutrients (minerals and vitamins) and fatty acids, and exhibit some health benefit activities including antimicrobial, antiviral, anticancer, and modulation of immune system. Milk proteins are also appreciated in food formulation for their dispersibility, solubility, and stability towards processing, their ability to form different types of gels, to stabilize emulsions and foams, their appearance, taste, and flavor. These properties allow the building of the large diversity of textures of dairy products and many other food products. In spite of their excellent functional properties, dairy product manufacturers sometimes replace part of milk proteins by other ingredients or additives in order to reduce the cost, improve stability, and extend the diversification of dairy product texture. These replacements grey over the image of naturalness conveyed by dairy products, a recent food quality concern expressed by the clean-label tendency. On a wider scale, it questions the way foodstuff is produced integrating traceability consideration, eco-conception approach, reflection about the use of ingredients or additives for healthier, more natural and environmental-friendly foods, without renouncing to other traditional food product quality criteria (security, practicability, pleasure, etc.). Research in the past decades has proven that whey proteins are highly versatile allowing their extensive modifications and assemblies in order to create unexpected new structures (nanotubes, fibers, spheres, etc.). The functionalities of these new structures in real food systems are not completely understood or even tested but this opens the door to putative new possibilities of using milk protein ingredients for extending their functional applications. They could allow the reformulation of food products matching better with consumer's expectations in term of nutrition, texture, taste, naturalness, durability, etc., and the advent of products echoing new usages and new consumption habits. In this review, we report on the most recent advances on the controlled modifications of whey protein structures for specific functionalities with a large focus on the research ongoing in our laboratory.

2 Whey proteins

2.1 Native whey proteins

Whey proteins describe the group of milk proteins that remain soluble after casein (CN) precipitation at pH 4.6. In this section, a brief overview of the structural characteristics of the main milk globular proteins is presented.

2.1.1 β -Lactoglobulin

β -Lactoglobulin (β -LG) is the major whey protein in bovine milk. Several genetic variants of β -LG exist, A and B being the most common, which only differ by two amino acids at positions 64 and 118; acid aspartic at position 64 and valine at position 118 of variant A are replaced in variant B by glycine and alanine, respectively. Hence,

each variant has 162 amino acids with a molecular weight (Mw) of around 18.3 kDa. β -LG molecular structure is well-established (Brownlow et al. 1997): basically, β -LG has 15% α -helix, 50% β -sheet, and 15–20% reverse turn. The β -strands (labeled A to I) are organized into two β -sheets facing each other and forming a flattened and conical barrel, called a calyx, as determined by X-ray crystallography (PAPIZ et al. 1986). This calyx constitutes an ideal binding site for hydrophobic ligands (Brownlow et al. 1997; Sawyer and Kontopidis 2000; Kontopidis et al. 2002; Le Maux et al. 2014). β -LG has 5 cysteines, 4 of which forming 2 disulfide bonds. The remaining free thiol group is buried within the protein structure on the β -strand H. The disulfide bonds contribute to the reversible denaturation of β -LG (Kitabatake et al. 2001), whereas the free thiol stabilizes the native protein structure (Jayat et al. 2004). At room temperature, β -LG exhibits pH-dependent reversible self-association behavior (Yan et al. 2013). At neutral pH (5.5–7.5), a stable noncovalent dimer is formed, whereas around its *pI* (5.2), β -LG exists as octamer. At pH below 3.5 and above 7.5, β -Lg is mainly monomer. Oligomeric state of β -LG is also sensitive to ionic strength, temperature, and the presence of hydrophobic ligands in the central cavity of the protein. Changes in β -LG structure upon temperature increase are reviewed in Qi et al. (1997) and De Wit (2009).

2.1.2 α -Lactalbumin

α -Lactalbumin (α -LA) is a α + β protein with a α -domain formed by four α -helix and a β -domain containing one anti-parallel β -sheet of three β -strands and a flexible loop. The overall structure is weakly organized with approximately 60% of unordered structure for 25–30% of α -helix and 10–15% of β -structure. The polypeptide chain has 123 amino acids for a Mw of 14.2 kDa and a *pI* between 4.2 and 4.5. α -LA has four disulfide bonds and one site of high affinity for calcium ($K_D=2.9 \times 10^8 \text{ M}^{-1}$; Hendrix et al. 2000) localized in the loop connecting the α and β domains. K_D varies when pH and temperature change. Below pH 5, the ability of α -LA to bind calcium decreases. Calcium stabilizes α -LA structure against heat-denaturation and accelerates its refolding (Forge et al. 1999). In the presence of calcium, the denaturation temperature of *holo* α -LA is 64 °C versus around 30 °C (Bernal and Jelen 1984; Griko and Remeta 1999) for the *apo*-form (calcium-free α -LA). At acidic pH, *apo* α -LA adopts a molten globule conformation. Under this conformation, *apo* α -LA forms microscopic aggregates when temperature increases beyond 50 °C.

2.1.3 Bovine serum albumin

Bovine serum albumin (BSA) is a helical protein (56–83% α -helix) with a molar mass of 66.4 kDa and a *pI* of 4.9 (Mattison et al. 1998). Among the 582 amino acids of the polypeptide chain, 35 are cysteine forming 17 intramolecular disulfide bonds and leaving 1 thiol group at position 34. These disulfide bonds involve only cysteine close to each others in the polypeptide chain, which is therefore organized in a series of short loops. BSA binds hydrophobic ligands such as fatty acids up to 4.5 fatty acids per BSA (Perez et al. 1989). At low ionic strength, BSA molecules self-assemble into small aggregates.

2.1.4 Lactoferrin

Lactoferrin (LF) is an iron-binding glycoprotein belonging to the transferrin family. The polypeptide chain is composed of 689 amino acids (Mw of 80 kDa) folded in two homologous lobes (N- and C-lobes) (Moore et al. 1997). Each lobe has two domains (N₁, N₂ and C₁, C₂) forming a cleft in between that is the binding site for one iron ion (Baker and Baker 2009). Iron increases the heat stability of LF and reduces its flexibility. LF is the main basic protein in milk (*pI* of 8.6–8.9). However, the charge distribution on LF surface is highly uneven (Baker and Baker 2009) and evolves according to the aggregation state of LF. Indeed, LF exists as ionic strength-dependent monomer-aggregate equilibrium (Chaufer et al. 2000; Mela et al. 2010). The equilibrium is shifted to the monomer at low ionic strength (and neutral pH); under this condition, LF molecules are positively charged. At higher ionic strength, LF forms small aggregates that are neutral (Chaufer et al. 2000) or negatively charged (*pI*~6) (Mela et al. 2010).

2.2 Structural characteristics of whey protein assemblies and related functionalities

While they are stable in native milk, the whey proteins are known to denature and assemble into aggregates upon heating at temperatures of ~60 °C or above in milk system (Dannenberg and Kessler 1988a). The aggregates are mainly formed through thiol-disulfide interactions and other noncovalent interactions (Donato and Guyomarc'h 2009; Morand et al. 2011b). The reaction can also be achieved at lower temperatures in concentrated systems, e.g., at 35 °C in ~500 g.kg rehydrated whey protein isolate (Zhou et al. 2008). In dairy technology, the reaction adversely affects the secondary phase of renneting during cheese manufacture because the whey protein aggregates interact with the casein micelles and prevent the fusion of the rennet gel (Guyomarc'h 2006; Anema et al. 2007). On the other hand, the presence of heat-aggregated whey proteins is desirable in yoghurt-making, where they have long been associated with a firmer, finer, and moisturized protein gel (Dannenberg and Kessler 1988b, c). Only 10% *w/w* of the total protein in the form of aggregated whey proteins, corresponding to about equimolar aggregates and casein micelles and to about 8% coverage of the surface of the casein micelle, suffices to significantly increase the final elasticity of acid milk gels (Andoyo et al. 2014).

Whey proteins are also reported to form a wide range of assemblies (fibrils, multistranded ribbons, spherulites, spherical particles, flexible strands, or fractals aggregates) depending on the conditions applied on heating (Nicolai and Durand 2013). Interesting perspectives are also presented by “switchable” particles of whey proteins, formed by coacervation of electrostatic-associated proteins that are reversible upon changes in the ionic environment and/or charge of the proteins (Bouhallab and Croguennec 2014). The versatility and the design of controlled structures from whey proteins essentially depend on the ability to tune the dimensions of the whey protein assemblies (shape and size), their internal arrangement (density and type of interaction involved), and the properties of the surface (surface charge and hydrophobicity). These structures offer applications in the control of food properties such as in the generation and stabilization of foams and emulsions, their use as thickening agents, or their use in the cold gelation, in the design of edible carriers for hydrophobic molecules like drugs,

oils, or for bacteria, in the generation of edible films, and coating for controlling mass transfer between media (Nicolai et al. 2011; Tavares et al. 2014).

2.2.1 Form factor, size, and density

Under diluted conditions, long-range electrostatic repulsions prevent whey proteins from aggregation. This steady state may be altered upon heating the solution above whey protein denaturation temperature. As a consequence, the polypeptide chains become more mobile and reactive amino acids (hydrophobic amino acids and cysteine) initially buried in the core of the protein structure become exposed on the surface and participate to protein-protein interactions. Protein concentration, time and temperature of heating, pH, and the presence of specific ions are the main factors affecting the dimensions of the assemblies. Some results briefly presented below on the dimensions of heat-induced assemblies of whey proteins were mainly obtained on studies conducted on β -LG and are extensively detailed in recent reviews (Nicolai et al. 2011; Nicolai and Durand 2013; Bouhallab and Croguennec 2014).

At low pH (pH < 2.5) and low ionic strength, conditions for which the protein net charge is high, whey proteins form fibrils when the solutions are heated at 80 °C and above for several hours (Fig. 1). Fibrils are long linear aggregates inside which the polypeptides establish mainly intermolecular β -sheets that are perpendicular to the long axis of the fibrils. The kinetics of fibril formation is slow and follows a nucleation/growth mechanism (Arnaudov and de Vries 2007). It is pointed out that a lag time during which proteins undergo some cleavages in the polypeptide backbone is required before fibril formation (Bolder et al. 2007; Akkermans et al. 2008). Under particular conditions, the fibrils of β -LG are also able to organize into larger structures such as helical ribbons (Lara et al. 2011) or spherulites (Domike and Donald 2007) (Fig. 1). Helical ribbons consist of lateral assemblies of fibrils whereas spherulites result from their radial assembly. Due to their symmetry, the spherulites exhibit a Maltese cross pattern when observed under polarizing light microscope. In the presence of salts, the spherulites grow larger and the inner core of the spherulite progressively loses its birefringence, probably because of distortion at the nucleation point of the fibrils (Domike et al. 2009).

In narrow ranges of pH (4.5–4.7 and 5.7–5.9) on either side of whey protein *pI* and at low ionic strength, stable suspensions of monodispersed spherical particles of about 200 μ m are obtained on heating (Schmitt et al. 2009) (Fig. 1). Between these two pH ranges, the spherical particles associate into large aggregates leading to a macroscopic phase separation or precipitate on aging. The same trend is observed at the pH of formation of the spherical particles when salt content of whey protein solutions is increased (Donato et al. 2009). More recently, it was observed that similar spherical particles of β -LG could be obtained at higher pH (up to pH 7.5) by a careful control of the Ca^{2+}/β -LG molar ratio, suggesting that the net charge of the native proteins (tunable by pH and Ca^{2+} content) is the crucial parameter for the formation of spherical particles (Phan-Xuan et al. 2014).

Small flexible strands with length of about 50–150 nm and diameter of about 5–10 nm are formed at low protein concentration and for pH conditions around 2.5–4.0 and >6.3 (Mudgal et al. 2009; Kavanagh et al. 2000). Randomly branched (fractal) aggregates resulting from the secondary aggregation of these small flexible strands are

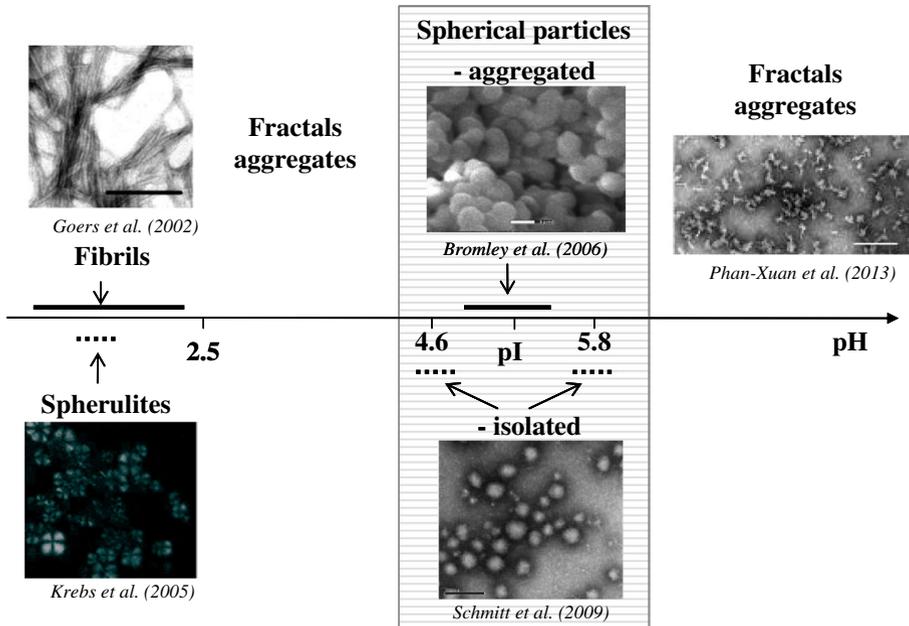


Fig. 1 Representation of the types of whey protein aggregates obtained on heating as a function of pH: Fibrils (reprinted from Goers et al. (2002) with kind permission from American Chemical Society); Spherulites (reprinted from Krebs et al. (2005) with kind permission from Elsevier); Isolated spherical particles (reprinted from Schmitt et al. (2009) with kind permission from American Chemical Society); Aggregated spherical particles, (reprinted from Bromley et al. (2006) with kind permission from Springer Science and Business Media); Fractal aggregates (reprinted from Phan-Xuan et al. (2013) with kind permission from American Chemical Society)

obtained at higher protein concentration (Nicolai and Durand 2013) (Fig. 1). The size of the aggregates increased continuously with protein concentration up to the critical concentration of gelation. The density of branching inside the fractal aggregates, and then the density of the aggregates, increases by increasing ionic strength due to the decrease in electrostatic repulsions between flexible strands (Baussay et al. 2004).

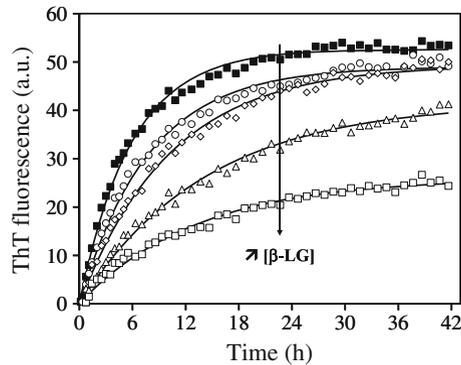
Formation of twisted fibrils was also reported at neutral pH after heating β -LG at 90 °C. Interestingly, addition of reducing sugar before heating in the same conditions slowed down the aggregation/fibrillation kinetics of heated β -LG, leading to a mixture of short fibrils and polydisperse aggregates (Pinto et al. 2012). In this study, we showed that glucose affected the growing step of covalent aggregates but not the initial denaturation/aggregation step of native protein. Concomitantly, we found that glucose affected also the aggregation kinetic of β -casein (β -CN) into spherical particles but in opposite manner as glucose promoted the formation of covalently linked spherical particles larger than those observed without glucose (Pinto et al. 2014). Additional structural studies could enlighten us on the exact chemical nature of crosslinking involved in the two types; β -LG versus β -CN aggregates. In parallel, β -LG aggregates produced in the presence of glucose, especially twisted fibrils, were shown to be more resistant to simulated gastrointestinal digestion (Pinto et al. 2014). However, the formation of such enzymatically resistant aggregates was induced under severe heating of isolated proteins (90 °C, 24 h), and their occurrence in formulated food needs to be

proved. Also, the functional properties of particles generated from both proteins with and without glucose deserve to be evaluated.

Dry-heating is another means used to denature and to form whey protein aggregates. Compared to heating in solution, the time of dry-heating for reaching a similar level of protein denaturation is usually longer. The size of the aggregates in dry-heated WPI increases (i) with the time of dry heating, (ii) with the pH increase from 2.5 to 6.5 of the concentrate used to prepare the whey protein ingredients for a fixed dry-heating time, (iii) and with the water activity (a_w) of powders (Gulzar et al. 2011, 2012). According to the experimental conditions of dry-heating (pH, a_w , time/temperature), different proportions of residual native proteins, soluble and insoluble aggregates, are recovered in the final powders (Gulzar et al. 2012). Enhanced heat-set gelling properties of reconstituted dry-heated whey protein powders at 10% (w/w) and pH 7 were found to be directly linked to a higher proportion of soluble aggregates. The maximal gelling properties were achieved earlier when powders were subjected to dry-heating at higher a_w and pH values (Gulzar et al. 2012). An optimized combination of the dry heating parameters is thus a good way to improve certain functional properties of whey proteins.

While many ingredients can be formulated using only the whey protein fraction, heating milk yields to co-aggregation of the whey protein and the κ -casein (κ -CN), essentially through thiol-disulfide interchange, with a ratio of 1–5 whey protein to 1 κ -CN molecule (Donato and Guyomarc'h 2009). Other caseins may be incorporated to a minor extent through noncovalent binding, especially in conditions that challenge the integrity of the casein micelle, e.g., elevated pH (Menard et al. 2005; Guyomarc'h et al. 2007a). Caseins have the ability to protect globular proteins from extensive heat-induced aggregation (Yong and Foegeding 2010). In β -LG solution at pH 7 containing the C-terminal part of the κ -CN, called caseinomacropeptide (CMP), a similar effect of CMP on the size of the aggregates of β -LG was observed even if the denaturation of β -LG was accelerated (Croguennec et al. 2014). The proposed mechanism is referred to as “chaperone” action of the casein or CMP upon the denaturing globular proteins. This action results mainly from the intrinsically unstructured and the well-separated hydrophilic and hydrophobic domains of the casein and CMP polypeptides (Farrell et al. 2006). In the case of κ -CN, which contains cysteins, it is proposed that thiol-disulfide interaction prevents the “chaperone” protein to dissociate and actually involves the casein into the aggregate. When heating model solution of whey protein isolates at 15 or 20 g/kg, Morand et al. (2011b) found that the hydrodynamic diameter of the resulting aggregates decreased from over 400 nm to ~100 nm when κ -CN was added prior to heating. The presence of as less as 5% w/w of the total protein as κ -CN is sufficient to divide the size of the aggregates by about twofold (Morand et al. 2011a). On the other hand, whey proteins reciprocally protect κ -CN (glycosylated and unglycosylated forms) from extensive fibrillation (Fig. 2); β -LG has a higher effect than α -LA. Hence, either the whey proteins or the κ -CN assemble into large structures (μ m scale) when heated in isolated form, but assemble into small aggregates (0.1- μ m scale) when heated together (Guyomarc'h et al. 2009; Morand et al. 2011a). Equally, the shape of the co-aggregated whey protein and κ -CN is intermediate between branched, fractal aggregates of whey proteins (Nicolai and Durand 2013) and elongated fibrils of κ -CN (Leonil et al. 2008). Reports describe ~100-nm diameter “noodle-like,” “round” or “oval-shaped” aggregates in milk when heated in various conditions that mainly

Fig. 2 Kinetic of the fibril growth of carboxymethylated κ -casein in the absence (*full symbol*) and in the presence of different concentrations of β -LG (20 μ M, *open circle*; 50 μ M, *open diamond*; 125 μ M, *open triangle*; 300 μ M, *open square*) monitored by the enhancement of thioflavin fluorescence intensity at 37 °C in 10 mM sodium phosphate buffer (pH 6). In all samples, κ -casein and thioflavin concentration was 50 and 240 μ M, respectively



affected the proportion of κ -CN in the aggregates and, hence, the propensity for anisotropic shape (for review, see Donato and Guyomarc'h (2009)). Finally, the density of the aggregates is also somewhat dependent on their composition. When either κ -CN or sodium caseinate was co-heated with whey protein isolate in model solutions, density of the resulting aggregates was found to be lower than that of whey protein-based aggregates (Guyomarc'h et al. 2009). The explanation may lie in the changes that were observed in the secondary structure of the proteins inside the aggregates, although only slightly dependent on the proportion of κ -CN. Increasing κ -CN in model aggregates resulted in lower levels of intramolecular β -sheets, α -helix, and/or disordered structures (Morand et al. 2011b). In contrast, when β -LG and CMP carry opposite charge (pH 4.0), CMP promoted the noncovalent aggregation of nonnative forms of β -LG (unfolded monomers and small aggregates) resulting in a rapid phase separation. Such phase separation did not occur in the presence of native β -LG, indicating that hydrophobic associations between CMP and residues exposed during heat-denaturation of β -LG molecules are involved (Croguennec et al. 2014).

Under specific pH conditions, whey proteins with opposite net charge, β -LG and LF or α -LA and LF, are shown to co-assemble in the absence of external energy input and form well-defined microspheres (Bouhallab and Croguennec 2014). The optimal pH condition for protein co-assembly is found when the molar charge equivalent ratio is reached. The driving forces of protein co-assembly are intermolecular electrostatic interactions and the release of water molecules or co-ions from the protein's surface (Bouhallab and Croguennec 2014). Similar mechanisms are responsible for the spontaneous assembly of LF with α -CN, β -CN, or κ -CN (Anema and de Kruif 2012). Nanotubes of α -LA of several micron length, and external and internal diameters of 20 nm and about 8 nm, respectively, are another form of protein self-assembly requiring low-energy input. To date, α -LA is the only protein proved to form such spectacular structure. The formation of nanotubes requires a limited hydrolysis of the α -LA backbone by a serine protease and under specific conditions such as the protein concentration, the presence of specific divalent cations, and the hydrolysis conditions, α -LA fragments self-assemble into nanotubes (Graveland-Bikker and de Kruif 2006). These reversible structures were suggested to be used as vehicles for the protection and the controlled release of bioactives.

2.2.2 Surface properties

Surface charge and apparent isoelectric pH values The apparent pI of whey protein aggregates is estimated at the interpolation to 0 of their electrophoretic mobility as measured by laser Doppler velocimetry. The pI of colloidal particles should depend on the salt phase used to suspend the aggregates, but Jean et al. (2006) reported that the mobility of aggregates present in the soluble phase of milk heated at 90 °C for 10 min does not depend on the ionic strength. They measured the pI of the natural serum aggregates suspended either in a milk ultrafiltrate or in a 20 mM imidazole/50 mM NaCl/5 mM CaCl₂ buffer or in the latter buffer diluted threefold in water and found $pI \sim 4.5$ at 25 °C. A value of 4.4 was also reported by Guyomarc'h et al. (2007b). At pH 6.7, in milk ultrafiltrate, aggregates have a ξ -potential of -17 to -19 mV that provided them some stability. When Jean et al. (2006) prepared artificial aggregates by heating a mixture of β -LG, α -LA, and κ -CN in a 20-mM buffer, the pI of the obtained aggregates was ~ 4.7 . The ξ -potential of the artificial aggregates in milk ultrafiltrate was ~ -20 mV at pH 6.7. This value is in the range of the values reported for unheated β -LG at pH 6.7 (Schmitt et al. 2009). After heat-treatment, the absolute negative charge of β -LG aggregates increased by about 10 mV compared to unheated β -LG. This increase is much more drastic at pH 5.7–5.9, the pH range of formation of stable suspensions of monodispersed spherical particles on heating, where a ξ -potential of -40 to -50 mV was reported (Schmitt et al. 2009). Below the β -LG pI , the charge of the aggregates is also maximum in the pH range of formation of stable spherical particles (pH 4.5–4.7), suggesting that the exceptional stability of the spherical particles is governed by electrostatic repulsions (Donato et al. 2009). At sufficient protein concentration, soluble aggregates formed on heating can associate together at room temperature and form a gel when their charges are shielded by adding salt or reducing pH close to pI of aggregate (Bryant and McClements 1998).

Another way to evaluate the influence of aggregate surface charge on the functional properties of dairy product is to modify preformed heat-induced aggregates with biochemical or chemical grafting as shown in the Fig. 3(D) (adapted from Famelart et al. 2011). Morand et al. (2011b) prepared soluble aggregates with a hydrodynamic diameter around 100 nm from whey protein solutions heated in the absence and in the presence of 15 to 40% w/w of κ -CN. These aggregates were very similar in terms of surface hydrophobicity, size and internal structure but differed regarding their pI . Pure whey protein aggregates had a pI of 4.7, as measured in milk ultrafiltrate, a value which decreased up to 4.3 with addition of 40% κ -CN in the mixture. This decrease in pI value is due to the respective pI values of whey proteins and κ -CN, i.e., 5.2 and 4.0, respectively. The ability of these aggregates to promote glucono-delta-lactone (GDL)-induced acid gelation of milk proteins at 35 °C in model systems containing 40 g.kg⁻¹ casein micelles and 10 g.kg⁻¹ aggregates suspended in milk ultrafiltrate was evaluated. The results showed a slight decrease in the gelation pH from 5.6 to 5.3 correlated with the decrease of the pI of the aggregates, without significant effect on the textural or microstructural properties of acid gels.

In another paper, Morand et al. (2012b) produced whey protein aggregates by heating whey protein solutions in the same conditions as used by Vasbinder et al. (2004), i.e., 68.5 °C for 2 h. Then the surface charge of the aggregates was modified by succinylation or methylation. The grafting of a succinyl group on the lysine

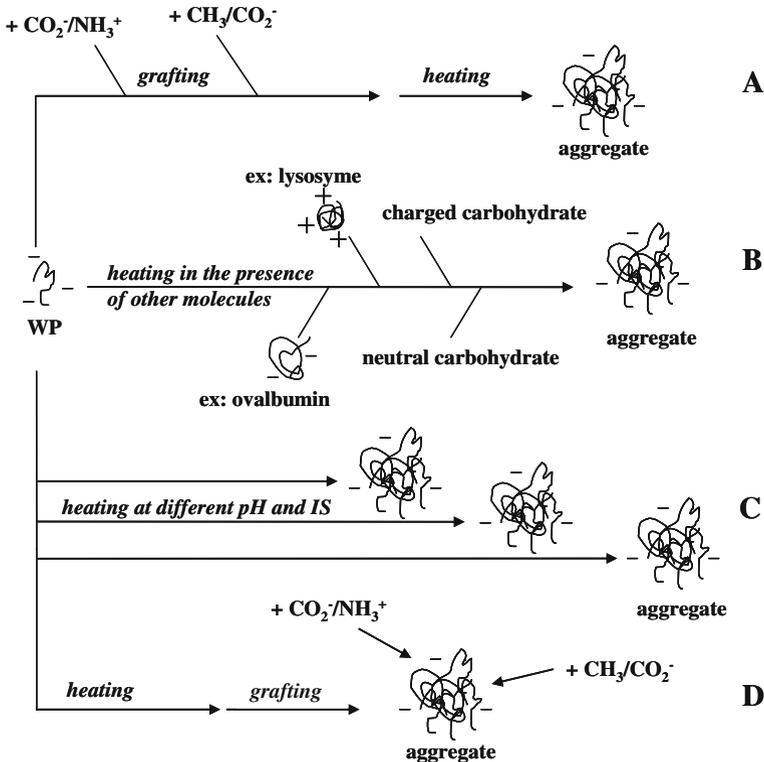


Fig. 3 Scheme of different available pathways to modify the isoelectric pH value (pI) or the charge of heat-induced whey protein aggregates. *A* by the chemical or biochemical grafting (succinylation, $+CO_2^-/NH_3^+$; methylation, $+CH_3/CO_2^-$) of whey proteins (WP) before heat treatment; *B* by heating whey proteins in the presence of other molecules (proteins with different pI ; neutral or charged carbohydrates); *C* by modifying the physicochemical conditions (pH and ionic strength (IS)) before the heat treatment; *D* by chemical or biochemical grafting of the heat-induced aggregates (adapted from Famelart et al. 2011)

residues or of a methyl group on carboxyl groups of proteins, respectively, reduced or increased the pI of the aggregates due to the substitution of a positive group by a negative one, or to the neutralization of a negative group, respectively. The modified aggregates had similar hydrodynamic diameter of around 130 nm but exhibited different pI ranging from 3.2 to 5.3. These authors tested the acid gelation of control and modified aggregates, alone or in mixtures with casein micelles, as previously described. They reported that the pI of the aggregates determined the pH of gelation in both studied systems, i.e., aggregates alone or mixed with casein micelles. Concerning textural properties of acid gels, an optimal pI of the aggregates at 4.0–4.4 was evidenced for maximal firmness of the gel; it is noteworthy adding that this is close to the pI of the natural serum aggregates found in milk heated at 90 °C for 10 min.

Surface hydrophobicity The surface hydrophobicity of whey proteins increases significantly upon heat-denaturation due to the exposition of hydrophobic patches on the surface of the unfolded molecular species. When their surface charge is not too high,

unfolded proteins and aggregates come close together and the exposed hydrophobic patches are able to initiate aggregation reactions. Like the surface charge, the surface hydrophobicity of the aggregates constitutes an important parameter to control their solubility and reactivity. Such modifications could have desired effects such as improvements of the foaming and emulsifying properties (Moro et al. 2001).

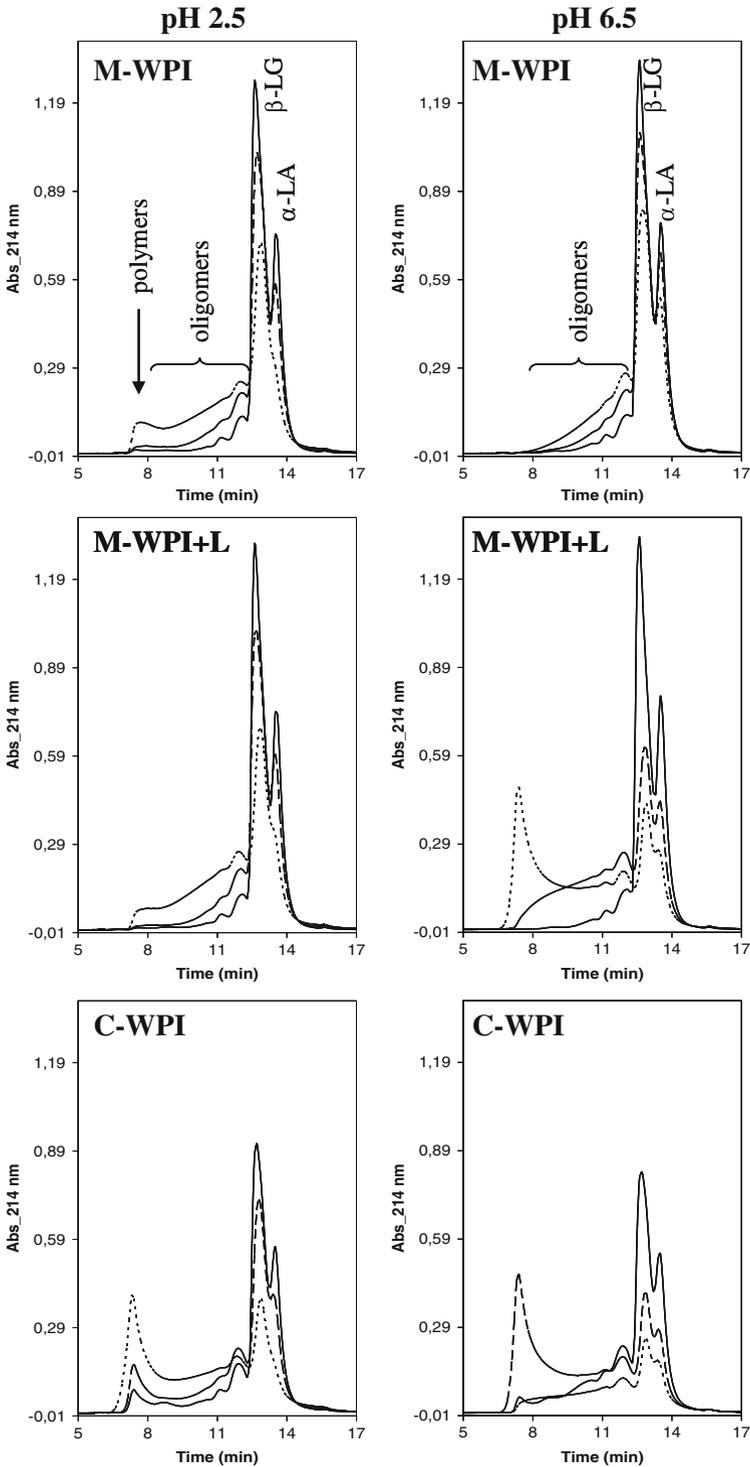
To evaluate the contribution of the aggregate's surface hydrophobicity on acid gelation of dairy mixtures, Morand et al. (2012a) produced heat-induced whey protein aggregates that were modified by grafting with chemical groups of ranged hydrophobicity. Whey protein aggregates were either succinylated using succinic anhydride or acylated using acetic, butanoic, or hexanoic anhydrides in order to obtain aggregates of increasing order of hydrophobicity. The hydrophobicity of aggregates was evaluated through their affinity for the nonionic 6-propionyl-2-(*N,N*-dimethylamino)-naphthalene (PRODAN) hydrophobic probe, by the ratio of the emitted fluorescence to the concentration of protein. It is expressed as the protein surface hydrophobicity or PSH in kg.g^{-1} . The whey protein aggregates exhibited surface hydrophobicity ranging from $\sim 3,000$ to $16,000 \text{ kg.g}^{-1}$. Such modifications also affected the net charge of the modified aggregates as the hydrophobic chemical groups were attached on lysine residues and some carried a negative charge. For low level of grafting, the hydrodynamic diameter of the modified aggregates was around 90 nm, against 120 nm for aggregates with high level of grafting. The acid gelation of suspensions of control (unmodified) or grafted aggregates, alone or in mixture with casein suspensions, was studied. An increase of the surface hydrophobicity of aggregates having the same *pI* (~ 3.7) induced a significant increase of the pH of gelation and of the maximum storage modulus G' of the acid gels made from suspensions of aggregates alone. With the most hydrophobic aggregates, gels began to form early after the addition of GDL and as soon as the value of pH reached ~ 6.0 . Concerning the mixtures containing aggregates and casein micelles suspended in milk ultrafiltrate, a limited increase of the surface hydrophobicity of the aggregates from $\sim 3,000$ to $6,000 \text{ kg.g}^{-1}$ induced an increase of the pH of gelation of the solutions, while for higher level of grafting, the gelation was too fast to measure correctly the gelation pH. Indeed, gelation occurred at $\text{pH} > 6.0$, pH range for which GDL induces a rapid pH drop. It is also for this limited increase in the surface hydrophobicity of the aggregates from $\sim 3,000$ to $6,000 \text{ kg.g}^{-1}$ that the G' dramatically increased, from 300 to $2,000 \text{ Pa}$. With the aggregates having larger hydrophobicity, from $6,000$ to $16,000 \text{ kg.g}^{-1}$, the storage modulus increases at the beginning of the acidification and then dropped, so that the final value of G' at $\text{pH} 4.5$ was lower, around 500 Pa . In the latter cases, the authors suspected an excessive contraction of the gel or syneresis as a significant whey separation was visible during rheological measurements. Moreover, increasing the surface hydrophobicity of the aggregates led to coarser acid mixed gels, with more clustered chains of particles and larger pore size. The authors assumed the effect of adding hydrophobic whey protein aggregates in acid casein gel was in some way similar to the effect of renneting. Indeed, renneting increases the surface hydrophobicity of casein micelles by releasing the hydrophilic C-terminal part of the κ -casein in the serum phase and creating hydrophobic patches on their surface. Similarly, the hydrophobic whey protein aggregates could first interact with the

Fig. 4 HP-GPC profiles of whey protein samples (M-WPI, model whey protein isolate obtained by mixing purified β -LG and α -LA in the same amount as in commercial WPI; M-WPI + L, M-WPI containing the same amount of lactose as in C-WPI; C-WPI, commercial WPI (Prolacta 95, Lactalis, France)) nonheated and dry-heated at two different pH (2.5 and 6.5). Before dry heating (*black line*), and after dry heating for 8 h (*dashed line*), and 24 h (*dotted line*). α -LA corresponds to α -lactalbumin monomer, while β -LG corresponds to the monomer and dimer equilibrium of β -lactoglobulin. Dry-heating was performed at 100 °C under $a_w=0.23$

micelle surface and create hydrophobic “patches” that could then lead to high attractive forces contracting and expelling whey from the gel. Combining the studies on the surface properties of the aggregates (charge and hydrophobicity), our group indicated that the aggregates are destabilized at higher pH when hydrophobicity and *pI* of aggregates increased, and allowed the gelation of whey protein aggregates alone or in mixture with casein micelles to happen at a higher pH value.

2.2.3 Type of interactions

Whey protein aggregates produced in solutions at neutral pH are stabilized mainly via intermolecular disulfide bonds and noncovalent interactions (Hoffmann and van Mil 1999). The balance between both types of interactions is tuneable by changing the ionic strength, pH, and the temperature of heating as indicated in the detailed review of De Wit (2009) on the thermal behavior of β -LG. Caseinomacropptide (CMP), which is present in most of commercial whey protein ingredients, also modified the structure of whey protein aggregates. Increasing the amount of CMP in β -LG solutions resulted in a decrease of the number of intermolecular disulfide bonds within the aggregates formed on heating (Croguennec et al. 2014). At low pH, the reactivity of sulfhydryl groups is reduced and the aggregates are mainly stabilized by noncovalent interactions. Interestingly, it was observed that disulfide bonds are the main intermolecular covalent bonds in the aggregates formed by dry heating of a mixture of purified whey proteins (80% β -LG and 20% α -LA) at pH value as low as 2.5. At pH > 4.5, intermolecular covalent interactions resistant to reducing agent, e.g., DTT, hence other than disulfide bonds, are also formed within the aggregates; their occurrence increased with increasing the pH for dry heating. These interactions are not identified yet but they are rather limited within the heat-induced aggregates formed in solutions. Similar results were obtained during the dry heating of commercial WPI (Gulzar et al. 2011) but the contribution of covalent bonds other than disulfide bond was enhanced in all range of pH studied (from pH 2.5 to 6.5). It is noteworthy that commercial whey proteins contain lactose. Whey protein ingredients with the lowest content of lactose still contain <4% (w/w) of free lactose on a dry matter basis. We observed by comparing dry heat-induced denaturation/aggregation of a mixture of purified whey proteins in the absence (M-WPI on Fig. 4) or in the presence (M-WPI+L on Fig. 4) of lactose that the presence of small amounts of lactose has a dramatic influence on the aggregates formed at pH 6.5, but not at pH 2.5. The different behavior of M-WPI+L compared to a commercial WPI (C-WPI on Fig. 4) during dry heating at pH 2.5 was explained by the fact that a part of the lactose molecules in C-WPI are bound to whey proteins before dry heating and the condensation products decomposed during dry heating. In the M-WPI+L samples, the condensation reaction of lactose with whey proteins is the limiting step and the subsequent reactions leading to aggregates are hindered. This suggests that



some of the intermolecular covalent bonds observed in dry-heated C-WPI involved the degradation products of Maillard intermediates such as α -dicarbonyl compounds, which are formed preferentially at pH 6.5 during Maillard reaction.

Milk acid gels can also be viewed as the stepwise assembly of, first, the whey proteins and caseins into aggregates upon heating and, second, (the connection) of these aggregates together upon destabilization of the milk by acidification. At each step, interactions are formed that may fall into two categories, namely covalent disulfide bonds that are rigid and noncovalent low-energy bonds that are weaker. Hence, it is expected that changes in the type of interactions involved on either step of the process may affect the final texture of the acid gel. A first approach is either to block or to release free thiol groups at the surface of preformed whey protein/ κ -CN aggregates in heated skim milk, in order to establish the importance of thiol-disulfide exchanges upon acid gel formation. Nguyen et al. (2012) verified that these treatments merely affected the protein assemblies in milk, i.e., the casein micelles or the aggregates, and did not affect their distribution between the serum and colloidal phases. When adding the thiol-blocking reagent *N*-ethyl-maleimide (NEM) into heated skim milk, Famelart et al. (2013) observed little change in the kinetic of formation and in final characteristics of model acid gels: the final elastic modulus of the gel did not decrease significantly upon the NEM addition; only the yield properties such as the firmness at large strain was significantly reduced. These results were confirmed by Nguyen (2014) and contradicted former results by Vasbinder et al. (2003) or Lucey et al. (1998) who reported decreased elastic moduli of acid gels using a similar approach. In fact, Lakemond and van Vliet (2008a, 2008b) reported opposite effects of the addition of NEM on the acid gelation of heated milk, in relation to the pH at which milk was heat-treated. When heated at pH 6.2, no effect of NEM addition appeared, while when milk was heated at pH 6.9, NEM addition leads to significant decreases in gelation pH and gel stiffness. Another explanation is that the amount of free thiols in heated milk is low, as discussed below, and blocking them could be insignificant on the decrease of the final elastic modulus of the acid gels. In contrast, when submitting preheated milk to a reductive reagent such as β -mercaptoethanol, Nguyen (2014) showed that the final elastic modulus of model acid gels can be increased from \sim 200 to 300 Pa. Furthermore, the yield stress of the gels increased, i.e., they resisted to larger deformation than control gels. These results support the idea that thiol-disulfide exchanges can occur during acidification, but are not necessary for the elastic modulus to reach values of hundreds Pa. The reason why blocking free thiol groups did not result in significant decrease of the final elastic modulus of the acid gels may be quantitative. If considering only the free thiol of β -LG in heated milk, engaged in thiol-disulfide interchanges (i.e., conservation of the free thiol groups), one can calculate that only about 0.2 mM of free thiols are present on the aggregates and can be blocked by NEM, which represents a small range of variation to study the effect of thiol-disulfide exchanges during acid gelation. On the other hand, the number of free thiols can be increased threefold by using reductive reagent such as β -mercaptoethanol, only considering κ -casein, which is preferably reduced in comparison to whey proteins (Nguyen et al. 2012, 2013a) and part of the β -LG (BSA being an even greater reservoir of cysteines). So, the range of thiol concentrations that can be explored when blocking the thiol groups is much lower than the one accessible when reducing disulfide bonds and hence, effects are more likely to be observed, if any. Figure 5 summarizes the

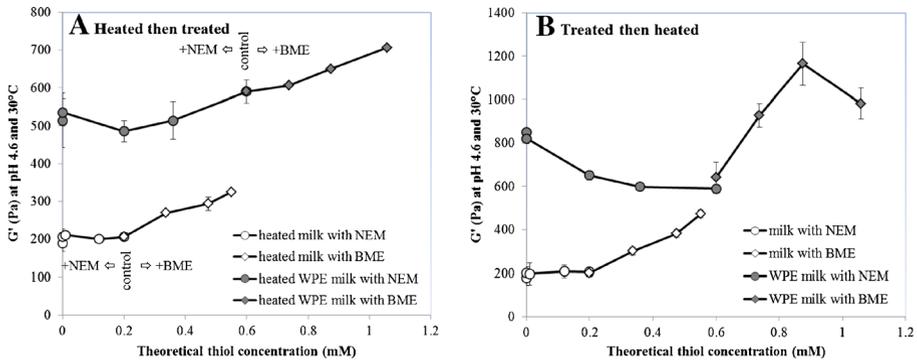


Fig. 5 Effect of the concentration of free thiol groups on the final elastic modulus, G' (Pa) of acid gels of heated (80 °C, 30 min) skim milk (milk, white symbols) or of heated (80 °C, 30 min) +1% w/w whey protein-enriched skim milk (WPE-milk, gray symbols) obtained after incubation with 2% w/w GDL at 30 °C for 3 h. The concentration of free thiol groups was modified using either NEM (circles) or β -ME (diamonds) after (a) or prior (b) heat-treatment. Figure adapted from the data by Nguyen 2014 (PhD thesis)

results obtained by Nguyen (2014) when submitting preheated skim milk or preheated whey protein-enriched skim milk (WPE-milk) to either NEM (to block the free thiol groups) or β -mercaptoethanol. It clearly shows that while blocking the 0.2 mM free thiol groups has little consequence on the final G' of acidified milk, blocking the free thiol groups of WPE-milk or releasing the free thiol groups on either sample can significantly increase the final firmness of the gels.

In order to modify the balance between covalent (strong) and noncovalent (weak) interactions in the gels, a second approach is to modify the nature of the bonds involved in the formation of the heat-induced aggregates themselves. To do this, thiols are either blocked or released using chemical reagents, prior to heating the milk (Fig. 5b). When thiol-blocking NEM is added to milk or milk supplemented with 1% w/w whey protein isolate, the irreversible denaturation of whey proteins on heating was decreased. Thus, up to 40% of the total whey proteins in skim milk and up to 80% in WPE-milk remained soluble at pH 4.6 after heat-treatment at 80 °C for 30 min. However, some heat-induced aggregates were formed and the final elastic modulus of the resulting acid gels was only decreased by 20–30% compared to milk without added NEM and subjected to the same treatment. The yield properties also changed as the gels were fractured more easily upon large deformation (Nguyen et al. 2014). These results indicated that the presence of disulfide bonds inside the heat-induced aggregates is important, but not essential, as milks containing thiol-blocking agent gave firm set acid gels of ~200 Pa (Fig. 5b, white circles). When NEM is added to milk supplemented with 1% whey protein isolate, the final elastic modulus of the acid milk gels was conversely increased (Fig. 5b, grey circles). Nguyen et al. (2014) attributed this effect to a conjunction of increasing number of heat-induced aggregates (due to the added WPE) and of increasing possibility for rearrangement during gel formation (due to the thiol-blocking agent). Also, they observed the dissociation of caseins in WPE-milk heated in the presence of NEM. κ -casein that is not covalently engaged to whey proteins in the presence of NEM has a higher propensity to stabilize the α - and β -caseins that dissociate upon heating, in the form of small nonsedimentable assemblies. A proportion of casein into dissociated form is suspected to enhance the firmness of acid milk gels.

To increase the number of free thiol groups in the milk prior to heating, Nguyen et al. (2012, 2013b) used up to 7 mM β -mercaptoethanol to reduce disulfide bonds. Interestingly, this treatment selectively affected the κ -CN, probably because this protein is found in polymeric form where the intermolecular disulfide bonds are more exposed to reactant than the intramolecular disulfide bonds of the globular proteins (Nguyen et al. 2012, 2013a). Upon heat-treatment of skim milks, supplemented with whey protein isolate or not, the presence of more free thiol groups on the κ -CN (reduced samples) was associated with the formation of larger aggregates, containing less κ -CN in proportion, and more largely associated with the surface of casein micelles than in control milks. It was proposed that the reduced κ -CN competed with the β -lactoglobulin to initiate the heat-induced aggregate formation, thus acting as an “anchor” to bind the whey protein to the casein micelle surface. Possibly, the number of sites where the whey protein could interact with the casein micelle increased as the κ -CN polymers were reduced. As the κ -CN was reduced, it became monomeric which accounted for its lower proportion in the aggregates, and hence was less able to be a “chaperone” to the denatured whey proteins resulting in larger-sized aggregates. In the aggregates, the proportion of β -lactoglobulin aggregated through disulfide bonds did not change upon β -mercaptoethanol addition and heat-treatment, but α -lactalbumin significantly engaged into more intermolecular disulfide bonds than in control milk. Acid gels made of milk preheated in the presence of β -mercaptoethanol exhibited more than twofold increase in the final elastic modulus compared to control, without significant effect on the gel microstructure (Fig. 5b, white and grey diamonds) (Nguyen et al. 2013a). Therefore, freeing thiol groups prior to heating proved a significant way to increase the final firmness of the gels, although the specific roles of the κ -CN, α -lactalbumin and β -lactoglobulin is still under investigation.

Besides the aggregates described above that contain covalent bonds, the microspheres formed by the co-assembly of whey proteins are stabilized only by noncovalent, low-energy interactions. Compared to the former that are stabilized by strong and irreversible intermolecular interactions, the microspheres are reversible when the surface charge of the protein constituting the microspheres are shielded by ionic strength or pH change. Understanding the mechanism of co-assembly and disassembly of the protein microspheres paved the way for the development of “switchable” carrier for selected molecules (Bouhallab and Croguennec 2014; Tavares et al. 2014).

3 Conclusion: perspectives

Heat treatment is still the predominant mean to create various structures from whey proteins even if plausible alternatives exist as indicated by the controlled self-assembly of α -LA into nanotubes or the co-assembly of LF and α -LA or LF and β -LG into microspheres. Much of the work has been done on model systems using purified proteins or group of proteins that differ significantly from real systems. The presence of other constituents may affect the building of whey protein suprastructures; this was illustrated by the impact of caseins or CMP on the size and shape of heat-induced whey protein aggregates, as well as the type of interactions maintaining proteins together. The addition of major cations such as sodium, calcium, and to a lesser extent potassium and

magnesium has been studied for a long time in order to modulate whey protein suprastructures and functionalities. In contrast, the effect of anions and trace elements was less investigated even though they could have a huge impact on the denaturation/aggregation reaction of whey proteins. For instance, copper induced the dimerization of denatured β -LG through oxidation of the free thiol groups (Bouhallab et al. 2004). The covalent dimers associate further into noncovalent aggregates, the size of which increased with increasing NaCl content in the medium. This gives suprastructures that differ significantly, at least by the type of interactions maintaining proteins together in the aggregates, from the ones obtained in the absence of copper (Gulzar et al. 2009). In the presence of molecules often recovered in milk protein ingredients such as fatty acids (Le Maux et al. 2012), or phospholipids (Le et al. 2007), the aggregation behavior of the proteins is changed on heating. These examples indicate that controlling the structure of whey protein aggregates is difficult in complex systems even though the structure of the building blocks (native whey proteins) is well known. In addition, processing used for the purification of milk protein ingredients often induce chemical and/or structural modifications such as lactosylation of milk proteins or the loss of water molecules in purified α -LA (Gulzar et al. 2013). To our mind, the influence of these modifications on the functional properties of whey protein ingredients is still underestimated. Their consideration could improve the control of formation of supra-molecular structure and the reproducibility of whey protein ingredient functionalities. Basic knowledge on the occurrence of such modifications and interactions should help innovation in the industry by continually offering new functional products.

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