Heating and glycation of milk proteins: aggregation and in vitro digestion

Michèle da Silva Pinto

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présentée par :

Michele DA SILVA PINTO

CHAUFFAGE ET GLYCATION DES PROTEINES DU LAIT : AGREGATION ET DIGESTION *IN VITRO*

soutenue le 13 Mars de 2013 devant la commission d'Examen

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To my grandmother Maria Rodrigues da Silva… in memorian.

"Better try and fail to worry and see life pass.
It is better to try, even in vain, to sit doing nothing until the end.
I prefer walking in the rain, sad days in which I hide at home.
I would rather be happy, but mad, according to live."
Martin Luther King

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ABSTRACT

Heating and glycation of milk proteins: aggregation and *in vitro* digestion

Milk proteins are important for their nutritional value and for their functional properties. Heat treatment is commonly used in dairy technology and in some cases milk is submitted to cumulative heat treatments. However, behavior of protein during heating depends on the physico-chemical conditions and also on the presence of other molecules (sugar, protein and lipid). We hypothesized that in addition of the well- known β-lactoglobulin (β-Lg) / κ-casein (κ-CN) complexes, other kind of aggregates occur in heated milk. To gain information of the occurrence of such species, the aim of this work was to study the effects of a strong heat treatment (90 ºC with different holding times) using following model systems: purified β-Lg and β-casein (β-CN), in presence of a reducing sugar (glucose) at neutral pH heated up to 24 h; and β-Lg with other milk proteins: α_{S2}-casein (α_{S2}-CN) and κ-CN at neutral pH. Furthermore, we studied the consequences on the *in vitro* simulated digestion of the produced aggregates. To characterize aggregates formed during heat-treatment and their breakdown, we use a series of techniques: gel electrophoresis (LDS-PAGE), thioflavin T essay, transmission electron microscopy, size exclusion chromatography, fluorescence, dynamic light scattering and hydrolysis kinetics. Our results revealed that high temperatures have favored the formation of covalent bonds, others than disulphide. β-Lg fibrils were produced at neutral pH and glucose decreased aggregate formation. On the other hand, glucose accelerate β-casein aggregation. About digestion, when we consider only Maillard Reaction, we show that proteolysis was impaired by glycosylation. When β-Lg was heated with α_{S2}-CN or κ-CN, we observed that homo- or hetero-aggregates were formed. Finally, the digestion of the mixed aggregates did not change when different proteins were heated together; even a slight increase of susceptibility to proteolysis was noted. These fundamentals studies highlighted some possible protein/sugar and protein/protein interactions during heat treatment, and give more details on the possible chemical transformations that occur during severe or cumulative heat treatments. Further, information about digestion of the aggregates may assist future studies about allergenicity or availability of milk proteins.

Keywords: heat treatment, whey protein, casein, aggregation, *in vitro* digestion

RESUME

Traitement thermique et glycation des protéines du lait: agrégation et digestion *in vitro*

Les protéines du lait sont importantes pour leur valeur nutritive et pour leurs propriétés fonctionnelles. Le traitement thermique est couramment utilisé dans la technologie laitière et dans certains cas, les composants du lait sont soumis aux effets cumulatifs de ce traitement. Le comportement des protéines lors du chauffage dépend des conditions physico-chimiques ainsi que de la présence d'autres partenaires (sucre, protéines et lipides). Notre hypothèse était que d'autres types d'agrégats peuvent se produire dans le lait chauffé, en plus du complexe entre β-lactoglobuline (β-Lg) et κ-caséine (β-Lg). Pour obtenir des informations sur la présence de ces espèces dans le lait chauffé, le but de ce travail était d'étudier les effets d'un traitement thermique intense (90 ºC, jusqu'à 24 h) à pH neutre sur les systèmes suivants: i- β-Lg et β-caséine (β-CN) purifiées, avec et sans glucose; ii- β-Lg en présences des caséines α_{S2} (α_{S2}-CN) ou kappa (κ-CN). En outre, nous avons étudié les conséquences sur la digestion simulée *in vitro* des agrégats produits dans ces conditions. Nous avons utilisé une série de techniques: électrophorèse sur gel (LDS-PAGE), microscopie électronique à transmission, chromatographie d'exclusion stérique, fluorescence, diffusion dynamique de la lumière et l'hydrolyse enzymatique. Nos résultats ont révélé que les températures élevées favorisent la formation de liaisons covalentes, autres que celles impliquant des ponts disulfures. Des fibres de β-Lg ont été produites à pH neutre et le glucose ralenti la formation des structures fibrillaires. Le glucose accélère en revanche l'agrégation de la β-CN. La glycation a eu pour conséquence de ralentir la cinétique de digestion des protéines. Des homo- ou hétéro-agrégats sont formés par chauffage de la β-Lg en présence de α_{S2}-CN ou κ-CN. Ces études fondamentales ont mis en évidence des interactions possibles de protéines/sucre et protéine/protéine pendant le traitement thermique, et donnent des indications sur les transformations éventuelles pouvant intervenir dans les laits chauffés.

Mots clés : Protéines du lait, glycation, agrégation, digestion *in vitro*

1. GENERAL INTRODUCTION

Milk is a complex biological fluid which is both an emulsion and a colloid suspension. The emulsion phase is constituted of milk fat globules within a water-based fluid that contains dissolved carbohydrates, minerals, vitamins, proteins and others small molecules. In this aqueous phase are dispersed colloidal particles: whey proteins dissolved as individual species at the molecular level and the caseins dispersed as large (50–600 nm) supramolecular structure named casein micelles (Fox, 2009; Walstra, 2006).

During milk processing, these proteins undergo different treatments involving high temperatures, exposure to oxidizing conditions and uncontrolled enzymatic or chemistry conditions. Among them, heat treatment is an essential operation in commercial processing of milk to provide acceptable safety, shelf life of dairy products or to improve the functional properties of final products (Jelen and Rattray, 1995). In some cases, milk can be heat several times before obtaining the final product, which means that milk can be submit to the cumulative effects of heat treatment. Heat treatment can promote damages in milk constituents, such as important changes in structure of proteins modifying the microstructure on the final product. These structural changes of the milk proteins lead to their denaturation and aggregation through covalent bonds, hydrophobic, ionic or Van der Waals' interactions. This process is strongly related with the physicochemical characteristics of the proteins, environment and processing conditions (Pellegrino et al., 1999; Zuniga et al., 2010). Under such conditions, milk proteins may aggregate with themselves or with other components such as carbohydrates. For the latter, the most prominent reaction is the formation of glycoconjugated proteins via the Maillard reaction, a nonenzymatic condensation of reducing sugars with free amino groups of protein by the formation of unstable Schiff's base. During the course of Maillard reaction are generated a wide large of compounds, including glycoconjugated but also protein-protein crosslink (Gerrard, 2002). It appears that many interaction pathways between components of milk can occur during heat treatments leading numerous possibilities to form aggregates between protein-protein and protein and other components. Due to the complexity of milk, numerous studies have been carried out on model systems using purified proteins to provide information when milk is heated; a known example is the formation of the complex between β-lactoglobulin and κ-casein which prevails in

heated milk (Cho et al., 2003; Bourais et al., 2006; Anema, 2007; Morand et al., 2011a,b). In spite of this extensive literature, it can be considered that numerous other aggregates can exist in heating milk and not yet have identified because they can be present at low concentrations, and/or difficult to characterize due the complexity of this medium. Our hypothesis was that other kind of aggregates with mixed proteins could be formed during milk heat treatment. The identification of these aggregates can allow having a better overview of heated milk and understanding how these aggregates can be formed, opens up opportunities to better control the milk processing during heat treatment.

In addition heat treatment can have profound effect on the nutritional properties of the final product. Formation of milk protein aggregates and products of advanced Maillard reaction could be responsible for changes to proteolysis sensibility. It was been shown that heat treatment led to differences in peptide patterns in course of *in vitro* digestion. This modification may change the allergenic potential of milk products (Dupont et al., 2010a, Corzo-Martinez et al., 2010).

SCOPE OF THE WORK

Our objective was to explore the heat-aggregation of β-lactoglobulin in presence of different partners (glucose as reducing sugar and other milk proteins) and its consequence on the *in vitro* protein digestibility using model systems with increased complexity: i) individual proteins with and without sugar and ii) mixed proteins.

In our study, we chose the following proteins:

- β-lactoglobulin (β-Lg), a whey protein with a single polypeptide chain of 162 amino acids and a molar mass of 18.3 kDa. It is a highly structured protein with high organization level.

- β-casein (β-CN) that constitutes up to 45 % of the casein of bovine milk. It is a single-polypeptide chain containing 209 residues with no cysteine residue.

- κ-casein (κ-CN) represents 8-15 % casein. It consists of 169 amino acid residues to a molar mass of about 19 kDa. Two cysteine residues give the possibility of disulfide bond formation.

- α_{S2}-casein (α_{S2}-CN) has 207 amino acids being 2 cysteine and a molar mass of 25.2 kDa

This work will be presented in two parties and three papers, which was preceded by a review literature:

Part (1): effect of glucose on the thermal aggregation of milk proteins

This was investigated for (i) β-Lg (paper 1) and (ii) β-CN (paper 2) with glucose. Comparison was conducted between β-CN, which has a loosely structure in contrast with β-Lg that has more compact globular structure (Kontopidis et al., 2004). We showed how glucose through Maillard reaction affected protein-protein interactions under severe heat treatment (90°C, different holding times). From kinetic studies and characterization of formed aggregates under these conditions, we shown that glucose have large impact on the formation of these aggregates. While glucose slows down the heat-induced aggregation of β-Lg at neutral pH, it accelerates the aggregation of β-CN. In addition, we show that proteolysis of aggregates formed during Maillard reaction was impaired by glycosylation.

Part (2): effect of caseins, α_{S2}-CN and κ-CN, on heat aggregation of β-Lg.

This part is presented in one paper (paper 3) that shows the interaction between β-Lg with α_{S2}-CN and β-Lg with κ-CN. These three milk proteins have free cysteine that can produce disulfide bonds inter or intramolecular and also cross linkages between different proteins. In our work, we confirmed this feature and further that high temperatures have favored the formation of covalent bonds, others than disulphide bonds. Moreover, the digestion of the aggregates was favored when proteins were heated together.

Finally, we presented a general discussion, followed by a general conclusion including prospects.

.

2. LITERATURE REVIEW

Heat treatments are commonly used in dairy process and have several purposes. The main purpose is usually preservation and extends shelf-life. However heat treatment, depending on the processing conditions, can result in irreversible changes in milk protein structure, leading to aggregation that may be followed by gelation. For example, in the milk system, heat treatment promotes preferential dissociation of κ-CN, from the micelles that forms mixed aggregates with β-lactoglobulin (β-Lg) (Anema et al., 2007). These aggregates are of prime importance to produce gels (cold and heat-set) in a wide variety of products such as yogurt (Nicolai et al., 2011). In the same way, protein can interact with reducing sugars during heating leading to the production of a wide range of compounds, including polymerized brown pigments (Gerrard, 2002). These changes may also be applied to improve the characteristics of products.

Moreover, dairy ingredients are also important for their nutritional value. Heat treatment can promote damages in milk constituents, such as important structural changes in proteins. Knowledge about these changes is important to reduce lost in nutritional quality of the final product. Besides that, the modification due to heat treatment can affect the allergenic potential of milk products (Dupont et al., 2010a).

The research with heat modified milk proteins is enhanced because the request of the dairy industry, the advent of new technologies and even the request to the human health such as knowledge about allergenicity, Alzheimer disease, diabetes and other troubles linked to the protein aggregation.

In this literature review, after a brief general presentation of milk proteins, focus will be on the thermal behaviour of milk proteins mainly those concerned by this work, β-Lg and caseins and its impact on their digestibility. Finally an overview is then given on the method for characterise protein aggregates.

MILK PROTEINS AND HEAT TREATMENTS

Cow milk contains approximately 3.3 % of proteins, of which 80 % are caseins which are the protein precipitated from milk at pH 4.6 and the remainder is mainly serum or whey proteins (Fox, 2009; Walstra, 2006)

There are two groups of proteins in milk:

- **caseins**. According to the homology of their primary structures, caseins are classified as α_{S1}-, α_{S2}-, β- and κ-casein. Now the caseins are also classified as

a member of the natively unfolded or intrinsically unstructured/disordered family of proteins (IUPs). The intrinsically unstructured/ disordered regions are linked to a distinctive amino acid composition, characterized by a high content of proline, glutamine, and glutamic acid and the depletion of cysteine. These proteins can exist either in a random coil conformation or in a conformation similar to a molten globule state, where some degree of secondary structure is preserved (Thorn et al., 2009). This limited folded structure plays an important role during heat treatment since caseins are stable and hardly change during heat treatment.

- **whey proteins.** The major whey proteins in bovine milk are β-Lg, α-Lactalbumin, serum albumin and lactoferrin (Farrel et al., 2004). In contrast to casein, whey proteins are highly structured (Kontopidis et al., 2004).

Heat treatment induces several events in milk during processing: denaturation, and aggregation of milk proteins, Maillard reaction, formation of lysinoalanine (Gerrard, 2002) dependant on the intensity of heat treatment (time/temperature). A major consequence of the heat treatment is the denaturation, aggregation including profound changes in interactions between the milk compounds.

Gerrard (2002) proposed a summary of the known protein crosslinking in foods including the effect of heat treatment (Figure 1).

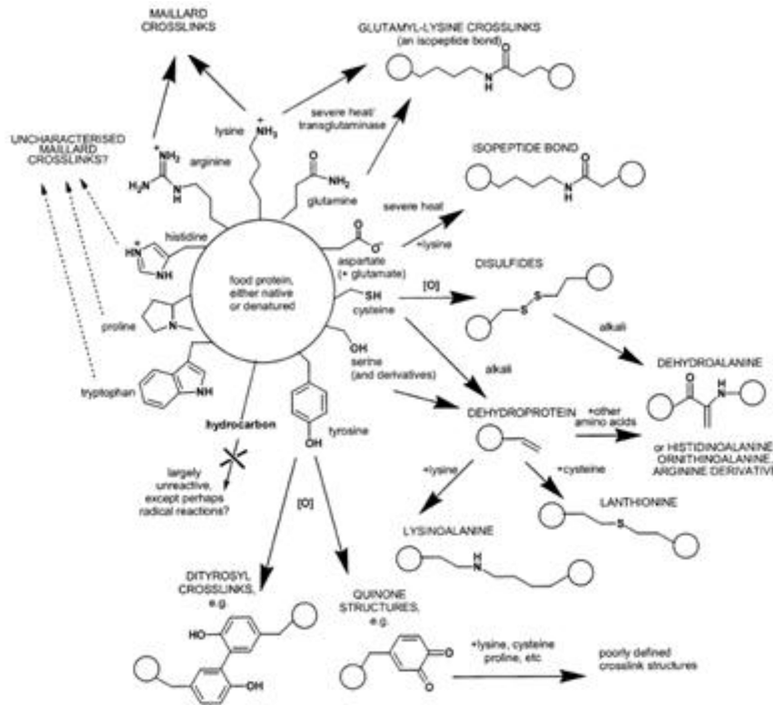

Figure 1: A summary of the crosslinking reactions that can occur during food processing. Source: Gerrard (2002) modified.

From this scheme, we clarify three types of covalent crosslinks : disulfide bridges, Maillard reaction and isopeptide bonds.

Disulfide bonds are the most common and well-characterized types of covalent crosslink in proteins in biology. They are formed by the oxidative coupling of two cysteine residues that are adjacent within a food protein matrix. (Gerrard, 2002). Large protein aggregates covalently linked by disulphide bonds was observed in milk samples heated at temperatures above 100 °C (Patel et al., 2006). About milk proteins, it is known that β-Lg contain two intramolecular disulfide bonds and one free sulfhydryl group and α-lactalbumine have 4 intramolecular disulfide bonds, while κ-CN and α_{S2}-CN have 2 cysteine residues (Fox and McSweeney, 2003). These cysteine residues allow the formation of intra-or intermolecular disulfide bonds.

In addition, milk protein can be modified by Maillard Reaction (MR) during heating. MR is a result from an initial reaction of a reducing sugar with an amino group (Martins and van Boekel, 2003). There are three stages of the MR: the initial, advanced and final stage. The initial stage of the MR, sugar reacts reversibly with the free protein amino-groups to produce a Schiff base which

undergoes an irreversible rearrangement to produce an Amadori product (Bourais et al., 2006). Upon prolonged heating (advanced stage), the Amadori rearrangement products undergo dehydratation an fission and yield colourless reductones as well as fluorescents substances, some of which may also be pigmented. The final stage is then the one in which most of the colour is produced. This stage is characterized by the formation of unsaturated, brow nitrogenous polymers and copolymers and although nitrogen-free polymers (Hodge, 1953).

This reaction may cause undesirable changes in some cases, such as browning in fluid milk, but otherwise, the reaction is desirable in dairy concentrated milk products such as "*doce de leite"* that are produced in South America, mainly in Brazil and Argentina, in a processing in which the milk is kept under heating at about 4-5 h.

Isopeptide crosslinks can be formed during food processing, via condensation of the ε-amino group of lysine, with the amide group of an asparagine or glutamine residue (Singh, 1991). These types of aggregates have been showed by Mudgal et al. (2011) as covalent bonds other than disulphide bonds during thermal aggregation of β-Lg. Moreover, they suggested that isopeptide bonds would be formed during purification or storage steps of proteins.

THERMAL AGREGATION OF β-LACTOGLOBULIN

To gain in comprehension about the mechanisms of heat-induced protein aggregation, β-lactoglobulin (β-Lg) has been used as a model system thanks to its availability in large amounts with a high purity grade and its well-known molecular structure.

β-Lg is the major whey protein in bovine milk. It is a single polypeptide chain of 162 amino acids with a molar mass of 18.3 kDa. In neutral pH it exists in the dimeric form (Figure 2).

Figure 2: 3D Structure of β-lactoglobulin Dimer

* PDB ID: 2Q2M. Vijayalakshmi, L., Krishna, R., Sankaranarayanan, R., Vijayan, M. An asymmetric dimer of beta-lactoglobulin in a low humidity crystal form structural changes that accompany partial dehydration and protein action. Proteins 71, 241-249, 2007.

The tertiary structure is recognized as a globular structure composed of nine anti-parallel β-sheets and one α-helix, which encloses a hydrophobic pocket as internal binding site and β-Lg contains two intramolecular disulfide bonds and one free sulfhydryl group. (Fox and McSweeney, 2003; Kontopodis et al., 2004). β-Lg aggregation in different time and temperature combination, concentration, pH, ionic strength, water activity in the model systems, with one or more proteins and also in complex systems such as skim or whole milk, whey protein concentrate (WPC) or isolate (WPI), with carbohydrate are strongly related (Cho et al., 2003; Anema and Li, 2003; Donato et al., 2007; Anema et al., 2007; Kethireddipalli et al., 2010; Lowe et al., 2004; Patel et al., 2006; Zuniga et al., 2010; Morand et al., 2011a,b; Burgos et al., 2012; Nguyen et al., 2012; Pinto et al., 2012).

In the heat β-Lg aggregation process, the mainly consideration is the disulfide bond interchanges. The heat treatment alters the β-Lg structure and the free cysteine (Cys121) initially reacts reversibly with the adjacent Cys106-Cys119 disulfide bond to give a free CysH119, which, in turn, reacts with the Cys66-Cys160 disulfide bond of the same or another β-Lg molecule to

give a free and reactive CysH160. This reactive β-Lg can react with disulfide bonds in other proteins (Creamer et al., 2004; Patel et al., 2006).

Nowadays, thermal behavior of β-Lg was studied. De Wit (2009) have presented a review, in which they showed the behavior of β-Lg heat treated between up to 150 ºC, and discussed research papers, that appeared until 2007. Figure 3, is a summary, proposed by De Wit (2009), which they presented what happens with this protein at pH > 6.8 between 20 and 150 ºC.

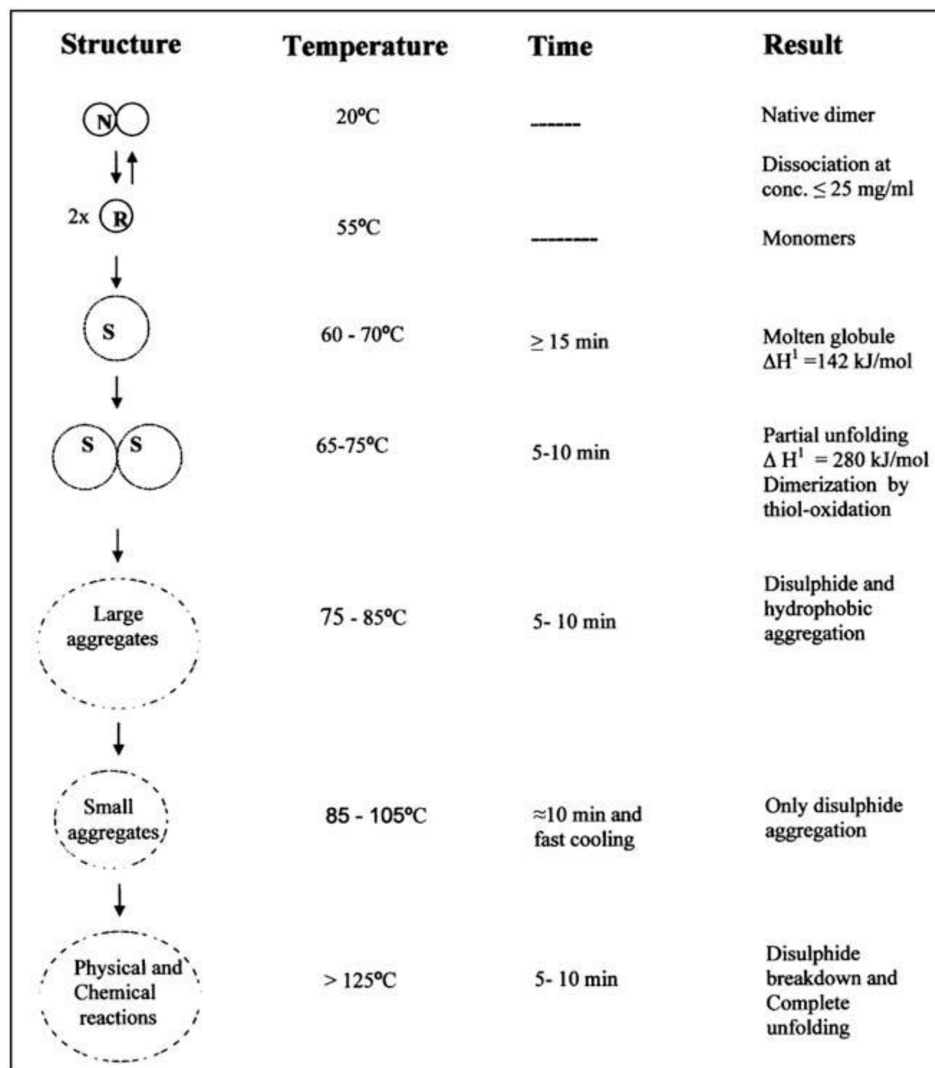

Figure 3: Schematic presentation of the proposed thermal behaviour of β-lactoglobulin at pH > 6.8 between 20 and 150 ºC. (N) Native conformation; (R) Reversible conformation; (S) Thiol group. Source: De Wit (2009).

Later, Nicolai et al. (2011) also published a review in which they have been investigated extensively the effects of pH, ionic strength and protein concentration on the formation and structure of heat-induced aggregates of β-Lg and further they have shown major trends. In this context, they described the last findings about β-Lg denaturation, kinetics and aggregation. Within, it is possible describe that at neutral pH, heated β-Lg solution is formed by stable fraction of denatured monomers and small oligomers that were aggregated rapidly when salt was added. At low concentrations, the oligomers are stable, however at higher concentrations they associate into larger aggregates. The aggregates are held together by both covalent bonds and noncovalent bonds. About the structure and aggregation mechanisms they have proposed heated β-Lg, at neutral pH, such as small curved strands, with a diameter of about 10 nm and a length of about 50 nm or between 15 and 27 nm of hydrodynamic radius (Rh). Above a critical association concentration the oligomers form larger primary aggregates with different shapes and sizes ($15 < Rh < 150$ nm) depending on the pH and the salt concentration.

Another interestingly feature was the long rigid strands that are formed at pH 2.0 that resemble so-called amyloid fibrils that are responsible for a number of neurodegenerative diseases (Jones et al., 2010).

In food processing, the impaired rennet clotting properties of heat treated milk was attributed to a synergistic effect among three individual milk components: the casein micelles with their heat modified surfaces, the serum whey protein/k-casein complexes, and other dialyzable serum components (Kethireddipalli et al., 2011).

Anema et al. (2004) reported changes in the viscosity of milk on heating, and that these changes are related to the size changes of the particles in the milk and the association of denatured whey proteins with the casein micelles. They showed that whey protein and casein micelles at pH 6.5 had high levels of interaction which was diminished when the pH was increased to pH 6.7. Along with, the viscosity and the size of the particles were decreased with this small increase in the pH of the milk (6.5 to 6.7). Donato and Dalgleish (2006) explained that heating milk at pH below 6.7 mainly caused whey protein denaturation and either bind to the casein micelles or to form complexes with the κ-casein of the casein micelles that then dissociate into the serum. When

the pH of heating increased from 6.7 to 7.3 the complexes amount increased but they are smaller in size and with a higher κ-casein/ whey protein ratio.

THERMAL AGGREGATION OF CASEINS

β-casein: β-CN has a molar mass of 24 kDa with a polypeptide chain of 209 amino acid residues. The presence of multiple proline residues, mainly in β-casein, affects the spatial organization of proteins, disrupting secondary structures like α-helix and β-sheet. The secondary structure consists of 10% α-helix, 13% β-sheets and 77% random structure (Fox and Brodkorb, 2008). The polar and nonpolar residues of the primary structure are grouped into sequences located far away from each other, which confers a character amphiphilic. β-CN is a highly calcium-sensitive phosphoprotein, displaying a pronounced self-association behavior (Portnaya et al., 2006). The attractive forces between the hydrophobic residues of the C-terminus of casein molecules are the driving force of the self-association. β-CN also exhibits temperature-dependent association behaviour; at low temperatures (<10-15 º C), monomers predominate, but as the temperature is increased, monomers associate, via hydrophobic bonding, into micelles (O'Connell et al., 2003). Ossowski et al. (2012) suggested that β-CN has a self-association behavior that is temperature-dependent at 6 and 25 °C.

Micellization of casein is characterized by the existence of a threshold concentration of monomers above which micelle formation is favourable, named critical micellization concentration (CMC). The CMC for β-CN ranges between 0.05 and 0.2% (w/v), depending on temperature, pH, and ionic strength. For example, Portnaya et al (2006) showed that the CMC decreased asymptotically from 0.15 to 0.006 mM as the temperature was raised from 16 to 45 °C.

κ-casein: κ-CN consists of a single chain of 169 amino acids, 2 cysteine residues. Approximately 60% of the chain is linked to at least six sugar molecules. The amino acid sequence shows a predominance of nonpolar residues at the N-terminal (1-105) while at C-terminal (106-169) there are predominantly polar residues (Farrell et al., 2004). κ-casein association is characterized by a critical micelle concentration which decreases as the ionic strength is increased. For example, it varies from 0.53 to 0.24 g / L for an ionic strength of 0.1 to 1.0 M. Polymerization of casein was observed for both the

oxidized and reduced forms, i.e., disulfide bridges (S-S) between casein molecules and with free thiol groups (SH). Cysteine residues in κ-casein allow the formation of intra-or intermolecular disulfide bonds (Fox and McSweeney, 2003). Hidalgo et al. (2010) proposed that during self-association, κ-CN opens its conformation by raising the exposition of hydrophobic regions to the medium. κ-casein can, apart from the known self-assembly into micelles, form amyloid-like fibrils at temperatures of 25 °C when subject to agitation (Ossowski, 2012) or 37 ºC during at least 1h of heating (Léonil, 2008).

α_{S2}-casein: α_{S2}-CN has 207 amino acids being 2 cysteine and a molar mass of 25.2 kDa. Circular dichroism and Fourier transform infrared spectroscopy of bovine α_{S2}-CN both report a 24 to 32% content of α-helix (Hoagland et al., 2001). Negatively charged residues are found clustered near the N-terminus and positively charged residues near the C-terminus: It is the most hydrophilic of the caseins. The presence of cysteine allows, inter or intramolecular, thiol-disulfide interchange reactions. Moreover, it is the most calcium sensitive and least abundant (10% or less) of the bovine caseins (Morris 2002; Fox and McSweeney, 2003, Farrell et al., 2004; Sgarbieri, 2005). The polymerization of casein is dependent of pH and the ionic strength of the medium, influenced mainly by electrostatic interactions, however is less affected by the temperature. Values beyond the maximum of ionic strength (0.2 M) are unfavorable for polymerization, because when the ionic strength increases, attraction force would be reduced and decreases the degree of association.

Furthermore, α_{S2}-CN upon incubation at neutral pH and 37 ºC (under physiological conditions) rapidly converted to twisted, ribbon-like fibrils with a beta-sheet core structure and the ability to bind amyloidophilic dyes such as thioflavin T (Thorn et al., 2008).

CONSEQUENCE OF HEAT TREATMENT ON THE NUTRITIONAL QUALITY OF MILK PROTEINS

The nutritive value of protein has two dimensions: quantitative, related to the total protein content in relation to energy or to weight and qualitative, related to its amino acid composition and the bioavailability of these amino acids. The milk protein composition and content differ in various dairy products as a result of the technology used in the manufacture (Fox and McSweeney, 2003).

In recent years, studies on how food is digested in the gastrointestinal tract have gained strength due beneficial or deleterious effects associated with its consumption. Increasing evidence shows that protein aggregation could lead to a modification of the protein resistance towards digestion (Mackie and Macierzanka, 2010; Dupont et al., 2010a;b; Bouzerzour et al., 2012; Corzo-Martinez et al., 2010; Barbé et al., 2013). These aggregates could be involved in the sensitization of patients suffering from allergy to cow's milk. It has been reported a mechanism of inhibition of uptake of these proteins by intestinal epithelial cells both *in vitro* and *in vivo* tests and a redirected uptake to Peyer's patches, with a modification balance of Th2/Th1 in mice associated with pro-inflammatory effects (Untersmayr and Jensen-Jarolim, 2006).

Numerous studies have reported beneficial effects associated with advanced Maillard reaction products, including antioxidative, antimicrobial, or antihypertensive activity (Finot et al., 1990). These properties give rise to a potential healthy food. On the other hand, attention has paid to the formation of milk protein aggregates and products of advanced Maillard reaction due to resistance to proteolysis. Milk processing led to differences in peptide patterns with an increase in the number of peptides found in digested samples. This modification can change the allergenic potential of milk products (Dupont et al., 2010a).

Overall, it may be inferred that most food allergens sensitize an individual via gastrointestinal tract, consequently digestibility and gut permeability are essential factors to consider since they may affect the allergenic potential. Recent studies are just beginning to reveal more about this issue.

AGGREGATES CHARACTERIZATION

The characterization of protein aggregates is made difficult they can be present with a wide range of different sizes, shapes, and composition depending on the conditions used. Nevertheless, it is essential to supply convenient methods that allow direct, rapid and cost-effective evaluation. In Table 1, we resumed the main techniques used to characterize the aggregates by their size, morphology or kinetics of formation.

Table 1: Main techniques used to characterize aggregates

Characterization	Technique	Characteristics/parameters	References that applied this technique in protein interactions study
Image analysis by microscopy	Transmission electron microscopy (TEM)	electrons penetrate a thin specimen and are them imaged by the appropriate lenses. The brightness of a particular area of the images is proportional to the number of electrons that are transmitted through the specimen.	Parris et al., 1997 ; Graveland-Bikkera and Kruifa, 2006; Zhu et al., 2010; Jones et al., 2010; Oboroceanu et al., 2010 ;
	Scanning electron microscopy (SEM)	produces an image that is a result of the secondary electrons generated by the electron scan of the surface of the specimen that are then detected by a sensor. The images produced give the impression of three dimensions.	Bozzola and Russel, 1999 Egerton, 2005; Murrieta-Pazos et al., 2012
	Atomic force microscope (AFM)	give the three-dimensional mapping surface specimen in the rigid surface either in air or immersed in a liquid. In this technique the sample need not to be electrically conductive.	Jones et al., 2010; Mezzenga et al., 2010; Oboroceanu et al., 2010; Doherty et al., 2011;
Relation with size nm/kDa	Dynamic light scattering (DLS)	measures Brownian motion by measuring the rate at which the intensity of the scattered light fluctuates when detected using a suitable optical arrangement. This measure is related to the size of the particles.	Donato and Dalgleish, 2006; Jean et al., 2006; Laos et al., 2007; Mahmoudi et al., 2007; Jachimska et al., 2008; Rullier et al., 2008; Gulzar et al., 2011; Kehoe and Foegeding, 2011; Moro et al., 2011;
	Electrophoresis	a technique for molecular separation. The separation can be made on a polyacrylamide gel (PAGE: polyacrylamide gel electrophoresis), placed between two flat sheets, by the relative size of the pores formed within the gel. The protein sample previously reacted with a detergent (normally sodium dodecyl sulfate (SDS)) and become negatively charged by their attachment to the SDS anions and should migrate at the same velocity towards the anode in free-flow electrophoresis. The separated protein bands can be seen by staining of the gel after electrophoresis running. The gel analysis can be qualitative or quantitative comparing with appropriate standards and scanning densitometry	Nguyen et al., 2012; Hong and Creamer, 2002; Jean et al., 2006; Anema, 2007; Patel et al., 2006; Patel et al., 2007; Broersen et al., 2007; Kethireddipalli et al., 2010 ;
	Size Exclusion Chromatography (SEC)	separates molecules according to differences in size as they pass through a gel filtration medium packed in a column. The sample is eluted isocratically (no buffers gradient) and molecules that are larger than the pores of the matrix pass through the column and leave it first. The entire separation is achieved when an equivalent to the volume of the packed bed passes through the gel.	Rojas et al., 2004; Donato and Dalgleish, 2006; Donato et al., 2007 ; Guyomarc'h et al., 2009; Corzo-Martinez et al., 2010 ;

Structural changes	Fluorescence spectroscopy	This technique evaluates changes in the emission spectra that often occur in response to conformational transitions, subunit association, substrate binding, or denaturation.	Pearce, 1975; Leclère and Birlouez-Aragon, 2001; Birlouez-Aragon et al., 2002; Alizadeh-Pasdar et al., 2004; Lakowicz, 2006; Broersen et al., 2007 ; Pinto et al., 2012; Morand et al., 2012;
	Fourier-transformed infrared spectroscopy	An infrared spectrum represents the absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms that formed the material. Each different material is a unique combination of atoms; no two compounds produce the exact same infrared spectrum. Fourier transformation and the desired spectral information are resulted by computations analysis.	Kauffmann et al., 2001; Patel et al., 2007 ; Oboroceanu et al., 2010 ; Iram and Naeem, 2012; Burgos et al., 2012;
	Circular dichroism (CD)	measure the response of differential absorption of left (L) and right (R) circular polarized radiation by chromophores which either possess intrinsic chirality or are placed in chiral environments. CD instruments, the spectropolarimeters, measure the difference in absorbance between the L and R circular polarized components and the CD spectrum as a function of wavelength.	Farrell et al., 2002 ; Cho et al., 2003 ; Khan et al., 2011;
Thermodynamic	Differential scanning calorimetry	a technique used in the studies of the conformational transitions of biological macromolecules, for example between the folded and the unfolded structure of a protein. This approach measures the excess heat capacity of the molecule of interest as a function of temperature.	Bengoechea et al., 2011; Kehoe and Foegeding, 2011; Zhou and Roos, 2011; Dickow et al., 2012; Murrieta-Pazos et al., 2012;
	Isothermal titration calorimetry (ITC)	The extent of the molecular interaction can be measured by the energy change when one of the compounds is titrated into the other. ITC methods can lead to a simultaneous determination of equilibrium constants (K_b) and enthalpy changes (ΔH) and this allow the full thermodynamic characterization, e.g. standard Gibbs energies and entropies.	Jung et al., 2008; Canabady-Rochelle et al., 2009; Taheri-Kafrani et al., 2010; Yuksel et al., 2010; Burgos et al., 2012;

Image analysis by microscopy

Important information about protein aggregation can be given by microscopy methods. Microscopy involves the study of the small objects that is not possible to examine by the unaided eye. In the electron microscopy an electron beam is focused and their very short wavelength allows the specimen to be imaged.

To promote the knowledge about protein aggregation, the microscopy techniques is used in many scientific works as an imaging support, to analyze the aggregate morphologies, however, either kinetic and nature of the interactions can be promoted by this technique. Graveland-Bikkera and Kruifa (2006) showed that α-lactalbumin nanotubes could withstand some important treatments, when they compared the TEM image of the unheated sample with the heated and freeze-drying samples. Zhu et al. (2010) used TEM images to report size and morphology of the whey protein/dextran conjugates. Jones et al. (2010) showed protein/polysaccharide morphologies of unheated and heated samples and the kinects of formation of the aggregates by TEM and AFM images. Oboroceanu et al. (2010) also used AFM and TEM to show the fibrillar aggregation process of β-Lg as function of the heating time. Moreover, we can cite Mezzenga et al. (2010) that showed the β-Lg fiber formation at different ionic strength and the evolution of fiber average diameter by AFM and Doherty et al. (2011) that applied AFM (to complete zeta potential analysis) to highlight electrostatic interaction during the formation of whey proteins micro-bead structures and the compatibility with integral probiotic bacteria. Studies were made using TEM to understand further the role of Ca^{2+} in the formation of soluble aggregates and insoluble precipitate in the whey milk protein systems based (Parris et al., 1997). Murrieta-Pazos et al. (2012) showed the SEM images of the four standards and agglomerated industrial milk powders. They analyzed the morphology with a focus on the surface differences.

Techniques related with aggregate size

Electrophoresis is a technique for molecular separation. This methodology is widely applied to characterize the milk protein. For example, Nguyen et al. (2012) used SDS-PAGE to give information on the distribution of

the proteins between the colloidal and serum phases or else the levels of proteins participating in disulfide bonds of the heated and modified milk samples. The amount of whey protein that interacted with the casein micelles in milk heated at different pH values was evaluated by electrophoresis as showed by Kethireddipalli et a.l (2010). Milk protein denaturation/aggregation was also evaluated by electrophoresis approach in some other works (Hong and Creamer, 2002; Jean et al., 2006; Anema, 2007; Patel et al., 2006; Patel et al., 2007; Broersen et al., 2007). Large protein aggregates covalently linked by disulphide bonds was observed in milk samples heated at temperatures above 100 °C (Patel et al., 2006), in the other words they observed the large aggregates electrophoresis bands (no reduced sample) with a consecutive decrease of the bands of whey proteins, k-CN and α_{S2}-CN bands. These proteins have free sulfhydryl groups. Moreover, when the samples were reduced with 2-mercaptoethanol, the aggregates bands disappeared with increase in the like-native protein bands.

Another separation method is chromatography, a physicochemical separation method for components in a mixture (Fontan et al., 2010). Different separation materials made different chromatography techniques. Size exclusion chromatography (SEC) separates molecules according to differences in size as they pass through a gel filtration medium packed in a column. The SEC results, as the chromatogram presented in Figure 4, can show the variation in concentration of the sample component, by absorbance in 280 nm, for example, eluted in the order of their molecular size. Molecules that do not enter in the matrix are eluted in the void volume (V_o) and those with full access to the pores usually elute just before the total column volume (V_t), but they do not separate from each other.

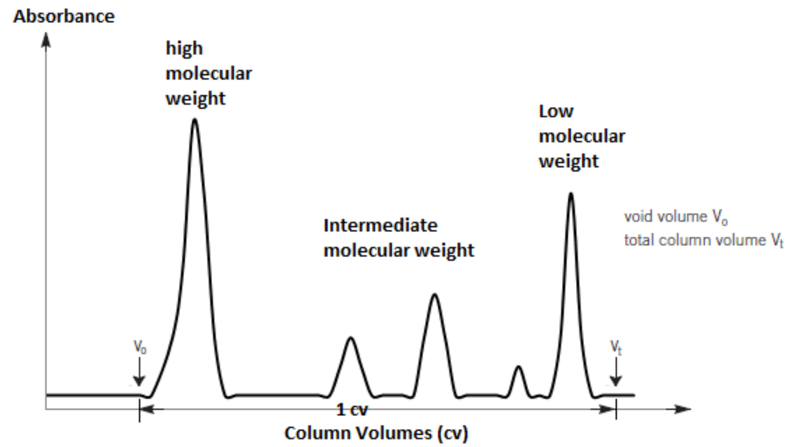

Figure 4: Theoretical chromatogram of Size Exclusion Choromatography

Among the SEC application can be cited purification proteins or molar mass determination and distribution analyses using a molar mass calibration curve. Rojas et al. (2004) reported this technique as final phase step purification for cheese whey proteins initially separated by polymeric and saline phases of aqueous two-phase systems. Corzo-Martinez et al. (2010) applied chromatography for the study of the aggregation degree of the heat β-Lg samples with or without galactose or tagatose. They showed that with increasing incubation time, formation of important levels of β-Lg cross-linked products took place, which was observed by increase of a peak near to void volume. This was an indication that glycation of β-Lg promoted its polymerization. In the other hand, Donato and Dalgleish (2006) performed SEC to give more explanation about the mechanisms of formation of soluble complexes in heat reconstituted skim milk in different pH. Donato et al. (2007), Guyomarc'h et al. (2009) and Morand et al. (2011b) also applied SEC to characterize whey protein/k-casein aggregates.

Dynamic light scattering (DLS), is a non destructive technique that yields a fast measurement of particle size in colloidal systems and then can give a proper distribution of heated milk systems. DLS techniques directly measure hydrodynamic quantities, a value that refers to how a particle diffuses within a fluid, usually the translational and/or rotational diffusion coefficients, which are then related to sizes (hydrodynamic radius) and shapes via theoretical relations (Pecora, 2000; Laos et al., 2007). For the diffusion of particles in dilute solution,

the hydrodynamic radius of the particle, Rh, may be obtained from the equations, as showed by Laos et al. (2007):

$$g^{(2)}\tau = 1 + \exp(-2D_t K_s^2 \tau)$$

$$\mathrm{K_s} = \frac{4\pi \mathrm{n}}{\lambda} \sin\frac{\theta}{2}$$

$$\mathrm{D_t} = \frac{kT}{6\pi\eta \mathrm{R_h}}$$

Follow the definition of the equations terms:

$g^{(2)}\tau$, scattered light intensity correlation function,

D_t, translational diffusion coefficient,

K_s, scattering vector,

n, is the refractive index of the solution,

θ, the scattering angle,

λ, the wavelength of light.

k, Boltzmann's constant;

T, absolute temperature;

η, is the solvent viscosity.

DLS was widely utilized to monitor the formation of soluble aggregates in heated milk and serum milk proteins in different environment conditions (Donato and Dalgleish, 2006; Jean et al., 2006; Mahmoudi et al., 2007; Rullier et al., 2008). As a exemple, Gulzar et al. (2011) calculated the average hydrodynamic diameter of soluble aggregates in whey protein isolate dry heated and Jachimska et al. (2008) determined the shape and dimension for globular protein's aggregates.

Structural characteristics

A technique used to identify changes caused by protein interaction is fluorescence spectroscopy. It is used by scientists from many disciplines as a research tool in biochemistry, biophysics, biotechnology, flow cytometry, medical diagnostics, DNA sequencing and genetic analysis, to name a few. Among biopolymers, proteins are unique in displaying useful intrinsic fluorescence. Phenylalanine, tyrosine, and tryptophan, the aromatic amino

acids are all fluorescent. A valuable feature of intrinsic protein fluorescence is the high sensitivity of tryptophan to its local environment. Changes in the emission spectra of tryptophan often occur in response to conformational transitions, subunit association, substrate binding, or denaturation (Pearce, 1975; Lakowicz, 2006). Caution should be taken when choosing a fluorescent probe for measurement some properties, like surface hydrophobicity, because their nature may cause overestimation due to the contribution of both hydrophobic and electrostatic interactions. This effect might be less significant in some proteins than others due to their isoelectric point as well as under pH conditions being investigated (Alizadeh-Pasdar et al., 2004).

Fluorescence already has been proposed for evaluating some milk protein modification. Leclère and Birlouez-Aragon (2001) evaluated the lysine damage upon heating in systems resembling heat-treated milk, composed of lactose and acetyllysine or protein mixtures by fluorescence of advanced Maillard products and soluble tryptophan as a rapid alternative for measuring furosine concentration, normally used to indicate lysine blockage. Birlouez-Aragon et al. (2002) proposed a fluorescent method, called FAST to discriminate the milk heat-treatments and estimate the resulting nutritional damage. The FAST method is a global approach for measuring the overall whey protein denaturation at excitation/emission 290/340 nm (tryptophan fluorescence) on the pH 4.6-soluble supernatant and the Maillard reaction at excitation/emission 330/420 nm (advanced Maillard products fluorescence). Pinto et al. (2012), also employed fluorescence approaches to show the evolution of advanced glycation end products in the β-Lg/glucose systems heated 90 °C over 24 h. Furthermore, they benefited of the changes in the fluorescence excitation spectrum due to interactions between amyloid fibrils and thioflavin T to verify if fibrils were formed.

In our study, we apply some methods to determine the morphology (shape and size) and follow the formation of aggregates. However, structural analyses were not made and can be considered themes to future studies. Some advantages/disadvantages of four techniques applied in our work, will be highlighted.

-TEM: this is a excellent technique for visualizing the sample. Moreover, protein solutions are samples relatively easy to prepare, since the following

steps are not necessary: fixing material, dehydration of the sample; permeation with resin to polymerization in a solid block following by cut the sample. The samples need only to be placed in thin reticulated metallic coated with thin polymer film and contrast enhancement step by immersing the sample solutions of uranyl acetate, for example. However, uranyl ions can bind to proteins, even if it should not bind to the specimen. An undesirable effect is to induce aggregation of the material. Furthermore, structural artefacts such as flattening of spherical or cylindrical structures are common due to deposition of heavy atom stains.

-LDS-PAGE: is a versatile, gentle, high resolution method for fractionation and physical-chemical characterization of molecules on the basis of size (Chrambach and Rodbard, 1970). However, beyond the fact that acrylamide is a toxic reactive (when in liquid state), we would not be able to use SDS-PAGE to separate two proteins with the same molecular weight from each other. Furthermore, the meshwork of fibers that form the polyacrylamide gel can block the proteins with high molecular weight to enter into the gel and this material could not be entirely analyzed.

-SEC: In this technique, molecules do not bind to the chromatography medium, which allows variation to the conditions to suit the type of sample or the requirements for purification. However, normally, to have a good resolution there has to be a 10% difference in molecular mass between the samples.

-Hydrodynamic diameter: this is a fast and accurate technique for monomodal suspensions. However, large particles scatter much more light and a small number of large particles can obscure the contribution from smaller particles. Furthermore, the diameter that is obtained by this technique is the diameter of a sphere and if the shape of a particle changes in a way that affects the diffusion speed, then the hydrodynamic size will change.

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3. β-CASEIN AND β-LACTOGLOBULIN: MAILLARD REACTION

Maillard reaction (MR) is a nonenzymatic condensation of reducing sugars with free amino groups of protein. MR is an important reaction that can take place in heated milk systems, leading to formation or inhibition of protein aggregates. β-casein (β-CN) and β-lactoglobulin (β-Lg), casein and whey protein, respectively, are important milk proteins not only for their nutritional value but also for their functional properties. The molecule of β-CN exhibits random structure, while β-Lg has a well-defined globular structure.

Our objective was for article 1 and 2 to explore and identify the possible interaction between β-Lg and glucose in investigating the modulatory effect of glucose on the formation of heat aggregates. The same approach was made for the system β-CN and glucose. We also investigated the consequences on simulated gastro-intestinal digestion. From kinetic studies and characterization of formed aggregates under these conditions, we show that glucose affected on the formation of heat aggregates. While glucose slows down the heat-induced aggregation of β-Lg at neutral pH, it accelerates the aggregation of β-CN. In addition, we show that proteolysis of aggregates was impaired by glycosylation. However for better understanding more resolving experimental techniques will be necessary, due to the heterogeneity of the final product.

During milk processing a variety of events can occur, such as protein aggregation, due to the high complexity of milk systems. In this context, studies with model systems are important to help understand the features of complex systems. Furthermore, changes in milk components during heat processing can be related with food illness or even with lost in nutritional value hence the interest to also understand how food is digested in the gastrointestinal tract.

PAPER 1: Glucose slows down the heat-induced aggregation of β-Lactoglobulin at neutral pH

Glicose retarda a agregação termo-induzida de β-Lactoglobulina em pH neutro

RESUMO

O comportamento da β-lactoglobulina (β-Lg) durante o tratamento térmico depende das condições do ambiente. A influência da presença ou ausência de açúcar redutor, na modificação da proteína durante o tratamento térmico foi estudada usando fluorescência, eletroforese em gel (PAGE), cromatografia de exclusão molecular (SEC) e microscopia eletrônica de transmissão. Produtos glicosilados foram produzidos durante 24 h à 90 ºC e pH 7. Os resultados de fluorescência revelaram um acúmulo de produtos avançados da Reação de Maillard e a formação de agregados durante o aquecimento. PAGE e SEC sugeriram que o produto nas amostras controle eram formadas essencialmente de agregados fibrilares ligados covalentemente. Estes agregados foram produzidos mais rapidamente que os produtos não glicosilados. Foi mostrado, que glicose interfere as etapas de crescimento dos agregados, mas não as etapas inicias de desnaturação/agregação da proteína nativa. A proteína modificada pela glicose formou uma mistura de pequenas fibras e agregados polidispersos. Estes experimentos revelaram a formação de fibras de β-Lg em pH neutro e a participação da glicose na desaceleração da formação de agregados.

Glucose ralentit l’agrégation thermo-induite de la β-Lactoglobuline à pH neutre

RESUME

Le comportement de la β-lactoglobuline (β-Lg) lors des traitements thermiques dépend des conditions environnementales. L’influence de la présence ou l’absence de sucre réducteur, à savoir le glucose, sur la modification de la protéine pendant le traitement thermique a été étudié à l’aide des techniques de fluorescence, l’électrophorèse sur gel (PAGE), la chromatographie d'exclusion moléculaire (SEC) et la microscopie électronique à transmission. Les produits glycosylés ont été formés pendant 24 h à 90 °C et pH 7. Les résultats de la fluorescence ont révélé une accumulation des produits avancés issus de la réaction de Maillard et la formation d’agrégats lors du traitement thermique. Les données de PAGE et de SEC ont suggéré que le produit présent dans les contrôles était composé essentiellement d’agrégats fibrillaires liés par des liaisons covalentes et que leur formation était plus rapide que pour les échantillons glycosylés. Nous avons montré que le glucose affectait l’étape de croissance des agrégats covalents mais pas l’étape initiale de dénaturation/agrégation de la protéine native. La protéine modifiée par le glucose a formé un mélange de fibrilles courtes et d’agrégats polydispersés. Ces expériences ont révélé la formation de fibrilles de β-Lg à pH neutre ainsi que le rôle du glucose dans la diminution de la formation des agrégats.

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Glucose Slows Down the Heat-Induced Aggregation of β-Lactoglobulin at Neutral pH

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ABSTRACT: The behavior of β-lactoglobulin (β-Lg) during heat treatments depends on the environmental conditions. The influence of the presence or absence of a reducing sugar, namely, glucose, on the modification of the protein during heating has been studied using fluorescence, polyacrylamide gel electrophoresis (PAGE), size-exclusion chromatography (SEC), and transmission electron microscopy. Glycated products were formed during heating 24 h at 90 °C and pH 7. The fluorescence results revealed an accumulation of the advanced Maillard products and the formation of aggregates during heating. PAGE and SEC data suggested that the products in the control samples were essentially composed of covalently linked fibrillar aggregates and that their formation was faster than that for glycated samples. We showed that glucose affected the growing step of covalent aggregates but not the initial denaturation/aggregation step of native protein. Glucose-modified proteins formed a mixture of short fibrils and polydisperse aggregates. Our results revealed that β-Lg forms fibrils at neutral pH after heating and that glucose slows the formation of these fibrils.

KEYWORDS: *β-lactoglobulin, heat treatment, aggregation, fibrils, Maillard Reaction*

INTRODUCTION

Heat treatment is an essential operation in commercial processing of milk to provide acceptable safety, shelf life of dairy products, or to improve the functional properties of final products.[1] Heat treatments induce structural changes of the milk proteins leading to their denaturation and aggregation. This process is strongly related with the physicochemical characteristics of the proteins, environment, and processing conditions.[2,3] In the presence of reducing sugars, the most prominent change is the formation of glycoconjugated proteins via the Maillard reaction, a nonenzymatic condensation of reducing sugars with free amino groups of protein via an unstable Schiff's base. This product can oxidize and condense with lysine or arginine residues to form glycated products and unsaturated brown nitrogenous polymers and copolymers that have not been fully characterized yet.[4,5] As a consequence of the heat treatment of milk, several chemical pathways are interconnected, inducing significant protein modifications through the establishment of covalent protein−protein interactions, mainly due to a rearrangement of disulfide bridges linked to the protein unfolding, glycation of proteins with high polymers, as well as β-elimination, condensation reactions, and partial protein hydrolysis.[6] So far, all these pathways and modifications are incompletely understood.

To gain insight into the mechanisms of heat-induced protein aggregation, β-lactoglobulin (β-Lg) has been used as a model system, thanks to its availability in large amounts at high purity grades and its well known molecular structure. β-Lg is a single polypeptide chain of 162 amino acids with a molecular weight of 18.3 kDa. Its globular structure is composed of nine anti-parallel β-sheets and one α-helix, and contains two intramolecular disulfide bonds and one free sulfhydryl group. β-Lg exists as a noncovalent dimer at neutral pH and dissociates into monomers at acidic pH.[7]

β-Lg forms several types of heat-induced aggregates whose morphology and size depend on the environmental conditions (concentration, pH, and ionic strength) and time−temperature couples.[8] At or near the isoelectric point, pI, spherical aggregates are formed, whereas far from pI, fibrils are observed: small curved strands at pH 7 and long rigid strands at pH 2.[8] In the presence of reducing sugars, the glycated β-Lg has been identified under different thermal conditions such as mild treatment (60 °C) by Morgan et al.[9] or at lower temperature and long time (40 °C for 24 h) by Corzo-Martinez et al.[10] Taken together, these results for β-Lg show the complexity of the mechanism of protein aggregation where multiple pathways can occur and small changes of environmental conditions can be critical in processing. The use of heat treatments is a current industrial practice in order to increase the functionality of dairy products. Considering the importance of the Maillard reaction, we have investigated how glucose affects the aggregation of β-Lg upon heating (90 °C for 24 h) at neutral pH to identify

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the different pathways and the complexes involved under these conditions.

■ MATERIALS AND METHODS

Materials. All of the chemicals and standards used were obtained from Sigma. Fresh bovine raw milk from homozygous cows was obtained from an experimental dairy farm (INRA, Rennes, France), and β-lactoglobulin was prepared using a modified method of Fauquant et al.,[11] as previously described by Leonil et al.,[6] in which membrane processes and low temperatures (below 56 °C) are involved to minimize chemical modifications, like the Maillard reaction. β-Lg A represented 98% of the protein in the freeze-dried powder based on reverse-phase high-performance liquid chromatography (RP-HPLC) analysis.

Glycation Experiments. Protein concentration was measured by the absorbance at 280 nm of samples centrifuged (30 min at 23,700g) and filtered through a 0.45 μM filter using a specific absorption coefficient of 16,730 L/mol/cm. Glycation was performed with glucose in aqueous system. Glucose−protein mixtures were prepared in sodium phosphate buffer 0.1 M, pH 7, containing 0.30 mM of β-Lg and 37.5 mM glucose, i.e., about 8 mols of glucose per mol of NH_2. Control samples were prepared as well without adding glucose. Model system solutions were heated at 90 °C for 24 h in Eppendorf tubes. After predetermined times between 0 and 24 h, the tubes were removed and cooled in an ice−water bath. Each sample was made in duplicate.

Characterization of Aggregates. *Protein Concentration.* The protein content of the solutions was determined by the Bradford method.[12] Experiments were carried out in 96-microwell plate format (Costar), with 200 μL of sample per plate on a Spectra Max M2 spectrophotometer (Molecular Devices, Sunnyvale, California). A pure β-Lg solution, whose concentration was determined by UV absorption at 280 nm, was used as a standard, at a range between 0 and 0.5 μM. All measurements were made in duplicate.

Advanced Glycation End Product (AGE) Fluorescence. The title process was according to the protocol described by Birlouez-Aragon et al.[13] except that experiments were carried out in 96-microwell black plate format. Fluorescence measurements were performed with 200 μL samples on a Spectra Max M2 fluorescence spectrophotometer (Molecular Devices, Sunnyvale, California). Excitation and emission wavelengths were 330 and 420 nm, respectively. When necessary, samples were diluted in pH 7 phosphate buffer.

Size Exclusion Chromatography (SEC). The separation was achieved by SEC using a BioSep-SEC-S4000 column, size 300 × 7.8 mm (Phenomenex, Torrance, USA), on a Waters system. Elution was achieved with 0.05 M sodium phosphate buffer, pH 7, at 0.5 mL/min for 30 min, and detection of eluting proteins was performed at 214 nm. The standard proteins used for calibration were apoferritin (481.2 kDa); BSA (66 kDa); and α-lactalbumin (14.2 kDa).

Gel Electrophoresis. The samples were analyzed by polyacrylamide gel electrophoresis (PAGE) using NuPage Novex (4−12%, Bis-Tris Mini Gels 1.5 mm, Invitrogen) under reducing and nonreducing conditions. Disulfide bonds were reduced by an overnight incubation in the NuPage lithium dodecyl sulfate (LDS) sample buffer containing 30 mM dithiothreitol (DTT) at room temperature. Samples containing approximately 12 μg of protein were analyzed. Gels were stained with Coomassie Brilliant Blue R250. A high molecular weight markers kit (3−200 kDa, Mark 12 Unstained Standard, Invitrogen) was used for calibration.

Transmission Electron Microscopy (TEM). Drops of 5 μM β-Lg suspensions were deposited onto glow-discharged carbon-coated microscopy grids. The liquid in excess was blotted with filter paper and a drop of distilled water was deposited on the preparation in order to rinse out the residual glucose and buffer salts. The water in excess was blotted, and prior to drying, the preparations were negatively stained with 2% (w/v) uranyl acetate. The samples were observed using a Philips CM200 microscope operating at 80 kV. Images were recorded on Kodak SO163 films.

Thioflavin T assay. Thioflavin T (ThT) can bind the amyloid fibrils by ionic and hydrophobic interactions changing the fluorescence excitation spectrum. An increase in ThT fluorescence intensity is related to a ThT bound along the length of the amyloid fibrils.[14] To perform the ThT fluorescence measurement, a ThT stock solution (0.63 mM) was prepared in phosphate buffer at pH 7 and stored at 4 °C.

To verify if fibrils were formed, experiments were carried out into a four-sided quartz cuvette: a working solution was prepared by diluting the ThT stock solution in phosphate buffer and adding it to the protein solutions so that the final dye and protein concentrations were 0.58 and 0.023 mM, respectively. Fluorescence measurements were performed on a Spectra Max M2 fluorescence spectrophotometer (Molecular Devices, Sunnyvale, California) at an excitation wavelength of 446 nm and an emission wavelength of 478 nm (475 nm cutoff). The net intensities were obtained after subtraction of the background signal. Results are expressed as the mean of five independent experiments.

■ RESULTS

The heat-induced aggregation of β-Lg was studied at pH 7 either in the absence or presence of glucose with a glucose/protein molar ratio of 125, which corresponds to an 8-fold excess of glucose over the amine groups of the protein.

Figure 1 shows the concomitant evolution of advanced glycation end products (AGE) and β-Lg denaturation-aggregation

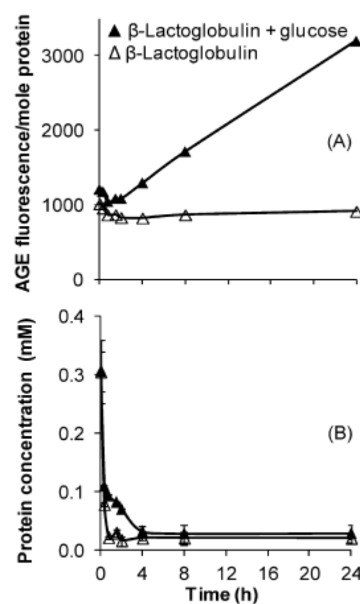

Figure 1. AGE fluorescence (A) and soluble protein at pH 4.6 (B) during heating of β-Lg solution at 90 °C, pH 7, with and without glucose. AGE were expressed per mole of protein.

after heating at 90 °C over 24 h with and without glucose. As expected, AGE formation increased with increasing heating time. After 4 h of heating, more than 80% of initial β-Lg precipitated at pH 4.6, a clear indication of denaturation and aggregation in the presence or absence of glucose. Before precipitation at pH 4.6, no precipitation was detected after 24 h of heating, an indication that the heat-induced aggregates were fully soluble at neutral pH in the presence as well as in the absence of glucose.

Native β-Lg exists as noncovalent dimers at pH 7 in equilibrium with native monomers.[15] The native β-Lg, as analyzed by SEC (Figure 2), is a mixture of monomers, prominent dimer forms, and minor oligomer forms. During heating

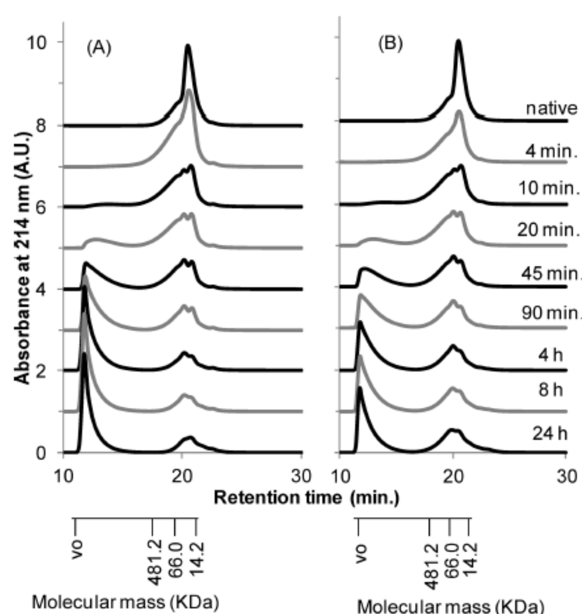

Figure 2. Evolution of SEC profiles of heated β-Lg samples (90 °C) without (A) and with (B) glucose. The holding time is indicated above each elution profiles. The secondary x-axis indicates the elution times of markers; vo = void volume.

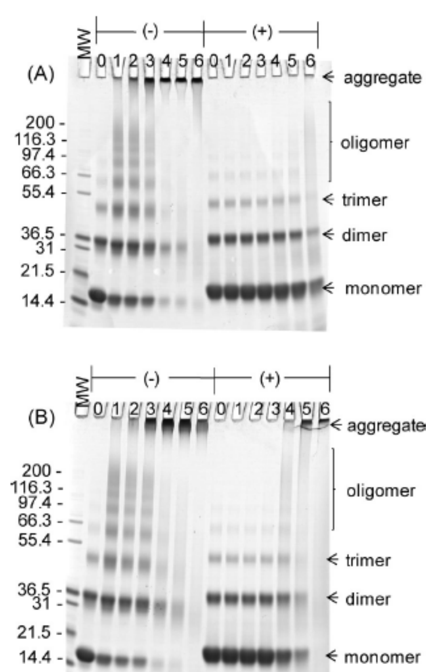

Figure 3. LDS−PAGE of heated β-Lg (90 °C, pH 7) without (A) or with (B) glucose before (−) and after (+) reduction with DTT. Native (0) and heated protein at different times: (1) 10 min, (2) 20 min, (3) 45 min, (4) 4 h, (5) 8 h, and (6) 24 h. MW = molecular weight marker.

and for both samples, a decrease of the peak height of native proteins (retention time = 21 min) and concomitant formation of high molecular weight products, eluted at the void volume (retention time = 12 min) were observed. Large aggregates eluted in the void volume started to be observed after 20 min heating, their amount getting significant after 45 min. The intensity of this peak was higher when β-Lg was heated without glucose. At the beginning of the heat treatment, the disappearance of species eluted at a retention time of 21 min was rather similar in the presence and absence of glucose. However, after 24 h of treatment, the high molecular weight species eluted at the void volume represented about 74% of the total product in the absence of glucose against 56% when glucose was present.

Nonreducing PAGE was performed to further characterize the β-Lg aggregates formed with and without glucose. Without glucose, a heating time-dependent decrease of the intensity of the initial bands (i.e., around 18 kDa for monomers, 36 kDa for dimers, and 54 kDa for trimers) was clearly observed, while oligomers and large aggregates progressively appeared at the top of the gel (Figure 3A). These aggregates did not enter the running gel and were the major species after 45 min of heating and above. As already shown by SEC analysis, the kinetic of disappearance of initial protein was not affected by the addition of glucose (Figure 3B). However, the addition of glucose affected subsequent aggregation steps, i.e., all the formed aggregates entered the electrophoresis gel and migrated as diffuse bands at the top of the gel.

To determine the nature of the aggregates formed during heating at pH 7, PAGE under reducing conditions was performed. The bands corresponding to oligomers (between 40 and 200 kDa) formed during heating disappeared in the gel for β-Lg heated both in the absence or presence of glucose. Comparatively, with no reduced samples, the intensity of monomer and dimer bands increased, indicating that most of these oligomeric species were made of monomers, held together by intermolecular disulfide bonds (S−S). These species seem to be the same for β-Lg heated in the absence and presence of glucose. The heat-induced large aggregates also disappeared after DTT treatment in the presence as well as in the absence of glucose. However, in the presence of glucose, some aggregated species still remained after reduction, indicating the occurrence of covalent bonds other than disulfide after long-time heating. Hence, the comparison of the electrophoretic profiles of β-Lg heated with and without glucose clearly shows the presence of new species for β-Lg heated with glucose.

We noted the presence of a dimeric form of β-Lg which progressively decreased during heating. This band was still present in unheated β-Lg under reduced conditions. This nonnative β-Lg dimer was not reduced by DTT whatever the experimental conditions tested (incubation time, DTT concentration) and therefore was not S−S-linked. This dimeric form could be attributed to the formation of covalent isopeptide bounds between protein molecules during purification or storage steps as already suggested by Mudgal et al.[16] These covalent dimers contributed to the aggregation process as their intensity decreased concomitantly to the increase of the intensity of large aggregates observed in the top of the gel.

β-Lg aggregates formed upon heating with and without glucose were observed by TEM. The morphological features of native β-Lg as well as samples heated in the absence and presence of glucose are clearly different (Figure 4). In the native sample, a mixture of aggregates of globular units and smaller individual objects were observed (Figure 4A). The presence of aggregated species in the native β-Lg TEM images is probably due to the effect of negative staining as no aggregates were detected by dynamic light scattering analysis of

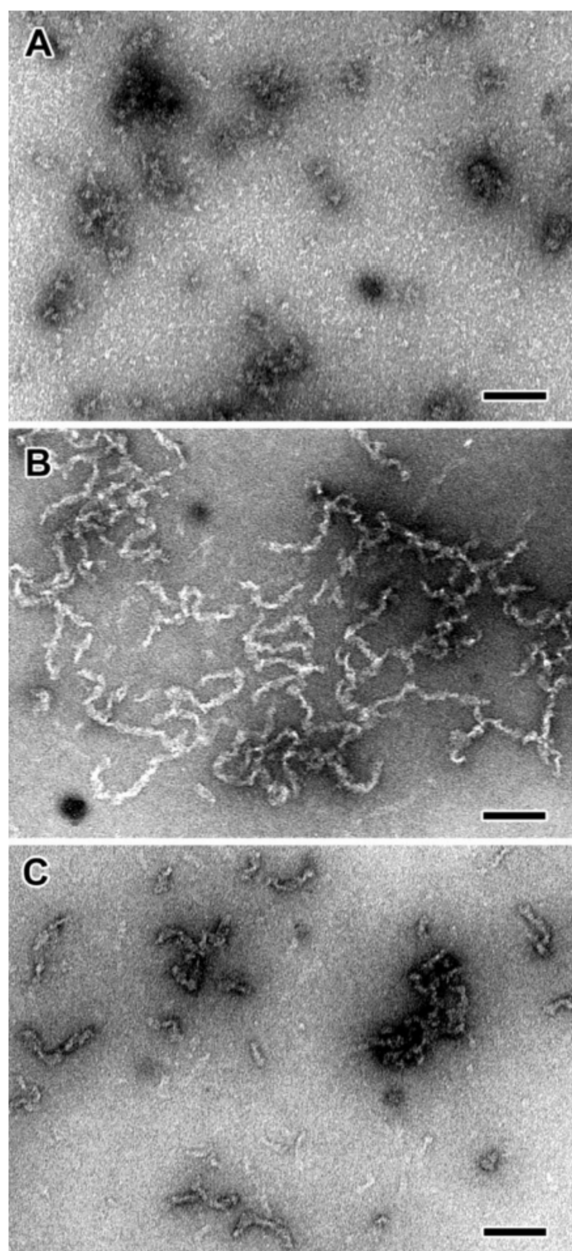

Figure 4. TEM images of negatively stained native β-Lg (A); heated for 4 h at 90 °C without (B) and with (C) glucose. Bars = 100 nm.

unheated β-Lg samples (data not shown). Images of the nonglycated sample treated at 90 °C during 45 min showed a mixture of aggregates and small fibril segments (not shown). The species seen in the native β-Lg sample almost disappeared after 4 h of heating, and in the sample heated without glucose (Figure 4B), a majority of twisted fibrils was observed. The TEM images of the β-Lg sample heated with glucose during 4 h showed a mixture of shorter fibrils (about 100 nm in length) and aggregates (Figure 4C). The width of the fibrils formed with or without glucose was constant and about 20 nm in both cases.

ThT fluorescence was used as a marker to identify the fibrillar nature of the aggregates and the kinetics of their formation. Figure 5 shows that the fluorescence intensity increased with time, with

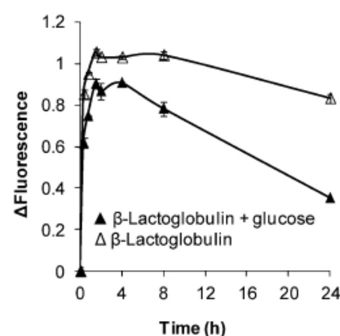

Figure 5. Thioflavin T (ThT) fluorescence of β-Lg heated at 90 °C and pH 7 both in the presence and absence of glucose. The emission intensity was measured at 478 nm upon excitation at 446 nm.

and without glucose, which is consistent with the TEM observations. The two curves have similar initial slopes, but the level at the plateau is higher in the absence than in the presence of glucose, indicating that glucose slowed down the initiation and/or the growth of β-Lg fibrils.

DISCUSSION

Nowadays, it is accepted, and more and more demonstrated, that the ability to form fibrillar structures is a common property of proteins. For a given protein, the formation of fibril specificity depends on the physicochemical conditions in relation to the proper protein properties. However, fibril formation has mostly been studied for pure proteins, and only few investigations focused on more complex systems containing, for example, additional molecules such as sugars and polysaccharides that can interact more or less specifically, more or less strongly with the proteins. To develop more applications, the challenge of ongoing studies is to better understand how protein fibrillation is affected by molecules or reaction that can be encountered in foods.

Many concepts and mechanisms of fibril formation from pure globular protein solutions were derived from studies on β-Lg, as the intimate mechanisms behind its denaturation–aggregation processes under a variety of experimental conditions are well described.[17,18] Electrostatic interactions have been proposed to be the main factor that controls the final morphology of heat-induced β-Lg aggregates. Variable particulate aggregates were formed under minimal electrostatic interaction, i.e., around the isoelectric point or at high ionic strength, while fibrillar structures developed under conditions favoring electrostatic interactions.[18] The morphology change as a function of pH was detailed by Jung et al.[19] using TEM. They showed that, after heating, the samples occurred as small curved strands at pH 7, large spherical particles at pH 5.8, and long semiflexible fibrils at pH 2. The structure and morphology of fibrils were shown to depend on other environmental parameters. However, the formation of pristine long β-Lg fibrils was promoted by a traditional thermal treatment at pH 2.[19]

Recent studies from different groups reported that the building blocks are small peptides derived from heat-induced hydrolysis rather than intact monomeric protein.[20−22] However, the exact nature of molecules leading to the pristine long fibrils after heating at pH 2 is still under discussion and probably depends on the exact heating conditions, as suggested by Jung and Mezzenga.[23] The fibrils were shorter and displayed a worm-like and granular appearance at pH 7 either in water[19]

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or in water–organic solvent mixtures.[24] Rather than fibrils, connected structural units containing visible β-Lg strands were observed when highly concentrated proteins (i.e., 8%) were heated at 85 °C at pH 7.[16] In our present work, we confirmed that β-Lg is able to fibrillate at pH 7 following heating. The twisted fibrils are long (up to 900 nm) with a width of about 20 nm. The shape of these fibrils is similar to that already reported at the same pH by Jung et al.,[19] but the overall dimensions are somewhat larger. The morphology that we observed may explain why some material was accumulated within the wells of the stacking gel during PAGE analysis (Figure 3). Interestingly, this means that the β-Lg fibrils were not completely dissociated by LDS detergent. These results are in agreement with those reported by Bolder et al.,[25] but they appear to contradict some previous studies that indicate protein fibrils (generally formed at pH 2) are easily dissociated and thus can enter PAGE.[16,21,26] The aggregates disappeared after DTT treatment indicating that covalent disulfide bonds are involved in the β-Lg fibrils formed at pH 7. The same conclusion was drawn by Jung and Mezzenga[23] to explain the isotropic–nematic transition found at pH 7 for β-Lg fibers formed at pH 2. They assumed that the higher isotropic–nematic phase transition may be related to the formation of covalent S–S bridges between neighboring fibers throughout activation of the free thiol groups favored at pH 7. However, covalent bridging of fibrils should probably be limited since randomly branched structures were not observed.

The addition of an excess of reducing sugar, namely, glucose, before heating induced the formation of AGE and influenced the β-Lg fibrillation process. This was revealed by SEC and PAGE analyses as well as by the shape of the aggregates observed by TEM. From a kinetics point of view, glucose did not seem to affect the initial step of protein denaturation–aggregation, corresponding to the initial disappearance of the native protein. In fact, the initial slopes of the disappearance of the native protein (SEC, PAGE) as well as that of the increase of ThT fluorescence are similar, regardless of the presence or absence of glucose. However, for longer heating time, glucose induced a significant decrease of ThT fluorescence. Similar decrease of ThT fluorescence was recently observed during heating of 10% β-Lg at pH 2 and high temperature (120 °C). Such decrease was attributed partly to local gelation or destruction of fibrils.[27] Given our LDS–PAGE results and the lower protein concentration used here, these assumptions do not seem to prevail in our case. We assume that nonfibrillar aggregation of worm-like fibrils could explain the observed decrease of ThT fluorescence. Glucose seems to decrease the growth rate of small aggregated species that accumulate, as shown by TEM images and PAGE. The progressive association of monomers into oligomers and then into small aggregates is clearly observed in the presence of glucose, as opposed to the rapid formation of very large aggregates in the control sample. The presence of glucose during heating clearly slows the formation of large molecular aggregates. A similar effect of β-Lg fibrillation by κ-carrageenan, an anionic polysaccharide, at pH 2 was recently reported.[28] Using TEM, AFM, and light scattering, the authors showed that κ-carrageenan complexes slowed the fibrillation kinetics probably through reduction of available protein monomers. Again, like in the control sample (see above), covalent bonds are involved in the aggregates. Interestingly, our results also show that part of the large aggregates is indeed resistant to LDS-reducing buffer (with DTT), even after a long incubation with a high excess of reducing agent. Consequently, disulfide and nondisulfide covalent bonds are involved in aggregated β-Lg in the presence of glucose. Several previous reports demonstrated that the formation of AGE throughout the Maillard reaction accelerated the fibrillation of amyloidogenic proteins such as β-amyloid peptide, human serum albumin, and τ-protein.[29−32] In contrast with these reports, we showed here that the presence of AGE slowed the formation of heat-induced β-Lg fibrils. Broersen et al.[33,34] reported that glycosylation improved thermostability of β-Lg at pH 7 but affects differently the aggregation behavior according to how the aggregation processes was induced: glucosylation was found to inhibit urea-induced aggregation, while on the contrary heat induced-aggregation was promoted.[33,34] However, the morphology of formed aggregates in both cases was not reported in this study. Our results are consistent with those of others showing a slow-down or even an inhibition of protein fibrillation after glycation and formation of AGE. Thus, according to Fernández-Busquets et al.,[35] only glucose favored the formation of globular oligomeric structures derived from aggregated species, while other tested carbohydrates promoted the formation of fibrillar structures. Lee et al.[36] showed that AGE inhibited the fibrillation of α-synoclein, an amyloidogenic protein. Interestingly, these authors showed that modified proteins predominantly exhibited nonfibrillar structures including spherical aggregates and atypical short fibrils. As was illustrated by the TEM image, heat treatment in the presence of glucose modifies the fibrillation process of β-Lg in a similar way, with a predominance of shorter fibrils (about 100 nm-long) mixed with spherical polydisperse aggregates.

The present results give new insight into the complex aggregation/fibrillation pathway of globular proteins at neutral pH and on how the process is affected by the Maillard reaction. From an academic point of view, our results could be explored for future understanding of controlled protein self-assembly processes, in particular the fibrillation process. Additional studies are in progress to identify the mechanism behind the observed changes and evaluate the nutritional and functional consequences. In fact, β-Lg and whole whey proteins are widely used as a functional ingredient. Our results showed that the presence of reducing sugar, as is the case in many whey protein powders, drive the aggregation kinetics and consequently the nature and morphology of heat induced protein aggregates. The use of these aggregates may be of interest to diversify textural properties, e.g., viscosity and shear thinning of formulated food products. These results are also relevant from a nutritional point of view because increasing demand exists for self-assembled structures that can be used for controlled delivery of bioactive substances.

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■ ABBREVIATIONS USED

β-Lg, β-lactoglobulin; PAGE, polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; RP-HPLC, reverse-phase high-performance liquid chromatography; AGE,

advanced glycation end products; LDS, lithium dodecyl sulfate; DTT, dithiothreitol; TEM, transmission electron microscopy; ThT, thioflavin T; pI, isoelectric point; SDS, sodium dodecyl sulfate; AFM, atomic force microscopy

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PAPER 2: Heating and glycation of β-lactoglobulin and β-casein: aggregation and *in vitro* digestion

Heating and glycation of β-lactoglobulin and β-casein: aggregation and *in vitro* digestion

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ABSTRACT

In the present work, we investigated the heat induced aggregation of two model proteins, β-lactoglobulin (β-Lg) and β-casein (β-CN), in the presence of glucose and consequences on simulated gastro-intestinal digestion. Protein aggregation and digestion were monitored using LDS-PAGE, size exclusion chromatography and transmission electron microscopy. Concomitant heating and protein glycation affect aggregation kinetics as well as protein sensitivity the digestion. Spherical covalently linked aggregates were favored in the case of β-CN in the presence of glucose. Glucose limited the formation of twisted fibrils from β-Lg. We clearly show that aggregates of both proteins obtained in the presence of glucose were more resistant to digestion. Those formed from β-Lg being highly resistant since still present after total simulated gastro-intestinal process. These findings underline the importance not only of the aggregation but also of the nature of formed aggregates on protein digestibility.

Aquecimento e glicosilação da β-Lactoglobulina e β-Caseina: agregação e digestão *in vitro*

RESUMO

Neste trabalho, estudou-se a agregação induzida pelo calor de duas proteínas modelo, β-lactoglobulina e β-caseína em presença de glicose e suas consequências sobre a digestão *in vitro*. A agregação das proteínas (90 ºC) e a digestão foram controladas utilizando as técnicas LDS-PAGE, cromatografia de exclusão molecular e microscopia eletrônica de transmissão. O aquecimento simultâneo e a glicosilação das proteínas alteram a cinética de agregação assim que a sensibilização das proteínas à digestão. Agregados esféricos ligados de maneira covalente foram favorecidos quando β-caseína foi aquecida em presença de glicose. Glicose limitou a formação pela β-lactoglobulina, de fibras com formato *twisted.* Pode-se mostrar claramente que os agregados das duas proteínas obtidos em presença de glicose foram mais resistentes à digestão. Sendo que os formados a partir da β-lactoglobulina foram fortemente resistentes, uma vez que eles persistiram após a digestão *in vitro*. Estes resultados colocaram em evidencia a importância não apenas da agregação propriamente dita, mas também da natureza dos agregados formados sobre a digestão das proteínas.

Chauffage et glycation de la β-lactoglobuline et de la caséine β*: agrégation et de digestion *in vitro

RESUME

Nous avons étudié l'agrégation induite par la chaleur de deux protéines modèles, la β-lactoglobuline et la caséine β, en présence de glucose et les conséquences sur leur digestion *in vitro*. L'agrégation des protéines et la digestion ont été suivies par LDS-PAGE, la chromatographie d'exclusion moléculaire et la microscopie électronique à transmission. Le chauffage concomitant et la glycation des protéines affectent la cinétique d'agrégation ainsi que la sensibilité des protéines à la digestion. Les agrégats sphériques liés de manière covalente ont été favorisés dans le cas de la caséine β en présence de glucose. Le glucose a limité la formation de fibrilles entrelacés de β-lactoglobuline. Nous avons clairement montré que les agrégats des deux protéines obtenus en présence de glucose étaient plus résistants à la digestion. Ceux formés à partir de la β-lactoglobuline sont fortement résistants, étant donné qu'ils restent présents après la simulation gastro-intestinale. Ces résultats soulignent l'importance non seulement de l'agrégation en tant que telle, mais aussi de la nature des agrégats formés sur la digestibilité des protéines.

INTRODUCTION

Cow milk contains approximately 3.3 % of proteins, of which 80 % are caseins which are the proteins precipitated from milk at pH 4.6 while the remainder is mainly whey proteins (Fox and McSweeney, 2003; Walstra, 2006). In bovine milk β-casein (β-CN) is the major casein amongst the four caseins identified (α_{s1}-, α_{S2}-, β- and κ-casein) and β-Lactoglobulin is the major protein in whey (Farrel et al., 2004).

β-CN and β-Lg are important not only for their nutritional value but also for their functional properties. The molecule of β-CN is flexible and exhibits a lot of segmental motion, but have little regular secondary structure and is close of a random structure. It has high propensity to form self-assembly into nearly spherical particles (Andrews et al., 1979; O'Connell et al., 2003). β-CN lacks cysteine residue and intermolecular bonds due to disulphide bond do not occur (Farrell et al., 2004). In contrast to β-CN, β-Lg that has a globular structure, composed of nine anti-parallel β-sheets and one α-helix, and contains two intramolecular disulphide bonds and one free sulfhydryl group (Kontopidis et al., 2004). The application of these proteins in the food industry requests a better knowledge of the properties of proteins as well as interactions with other food components (Nacka et al., 1998).

Heat treatment is one of the most widely used unit operation in the food industry. Before obtaining final product, the raw material can undergo cumulative thermal treatments, which modify the microstructure in the final product. This extensive heat treatment can promote numerous types of interactions between different milk components. Maillard reaction is an important reaction that can take place in heated milk systems. It is described as a nonenzymatic browning which involves the reaction of carbonyl compounds, especially reducing sugars, with amino acids and proteins (Brands and van Boekel, 2001). In our previous report (Pinto et al., 2010) we showed the effect of glucose on the aggregation behavior of β-Lg at neutral pH.

In the present study, we investigated how glucose can affect the aggregation of β-CN, a random protein compared to β-Lg, a globular protein. Formation of milk protein aggregates and products of advanced Maillard reaction could be responsible for changes to proteolysis. This modification can modify the allergenic potential of milk products (Dupont et al., 2010a). Hence,

we studied the resistance of aggregates formed to *in vitro* digestion. We focused on the characterization of aggregates using a series of methods such as size exclusion chromatography, dynamic light scattering and transmission electron microscopy as well as electrophoretic methods, the latter one used both for following kinetics of formation and digestion of aggregates.

MATERIALS AND METHODS

Materials. All the chemicals and standards used were obtained from Sigma. Fresh bovine raw milk from homozygous cows was obtained from the experimental dairy farm (INRA, Rennes, France). The method described by Fox and Guiney (1972) was used to prepare β-CN that was further purified by ion-exchange chromatography according to a modified method of Guillou et al. (1987). β-Lg A was prepared from the milk of homozygous cows as reported by Leonil et al. (1997), in which membrane processes and low temperatures (below 56 °C) are involved. The freeze-dried β-LG A have a purity of 98% as determined by reversed-phase HPLC analysis. Protein concentration was determined by measuring the absorbance at 280 nm, using extinction coefficient values of 0.458 and 0.937 g/L/cm for β-CN and β-Lg, respectively.

Glycation experiments. Glycation was performed with glucose in aqueous system. Glucose-protein mixtures were prepared in sodium phosphate buffer 0.1 M, pH 7, containing 0.30 mM of β-Lg, 040 mM of β-CN and 37.5 mM glucose, *i.e.*, about 8 moles of glucose per mole of NH_2. Control samples were prepared as well without adding glucose. Model system solutions were heated at 90°C for 24 h in 2 mL *Eppendorf* tubes, that were removed in predetermined times between 0 and 24 h, and immediately cooled in an ice-water bath. Each treatment was made in duplicate.

***In vitro* aggregates digestion**. We followed the method described by Dupont et al. (2010b) with modifications. Porcine gastric mucosa pepsin (activity 3.802 U/mg of protein) was added to give 182 U of pepsin/mg of β-Lg or β-CN (0.1 mM, final concentration). Aliquots (100 µL) were removed over the 60min digestion time course. Pepsinolysis was stopped by raising the pH using 0.5 M sodium bicarbonate. Before duodenal proteolysis, the pH of samples was adjusted to 6.5 by addition of 0.1M NaOH and the following components

(duodenal digestion) were added to give final concentrations: 4 mM sodium taurocholate, 4 mM sodium glycodeoxycholate, 26.1 mM bis-Tris buffer pH 6.5, 0.4 U/mg of β-Lg or β-CN bovine α-chymotrypsin (activity 52.6 U/mg of protein), 34.5 U/mg of β-Lg or β-CN porcine trypsin (activity 13.390 U/mg of protein). Aliquots (100 µL) were removed over the 30 min digestion time course, and proteolysis stopped by addition of a twofold excess of soybean Bowmann–Birk trypsin-chymotrypsin inhibitor above that calculated to inhibit trypsin and chymotrypsin in the digestion mix.

Characterization of aggregates and digested proteins

Gel electrophoresis. The samples were analyzed for polyacrylamide gel electrophoresis (PAGE) using NuPage® Novex® (4-12%, Bis-Tris Mini Gels 1.5 mm, Invitrogen™) under reducing and non-reducing conditions. Disulfide bonds were reduced by an overnight incubation in the NuPage® Lithium Dodecyl Sulphate (LDS) sample buffer containing 30 mM Dithiothreitol (DTT) at room temperature. Samples containing approximately 12 µg of protein were analyzed. Gels were stained with Coomassie Brilliant Blue R250. A high molecular weight markers kit (3-200 kDa, Mark 12™ Unstained Standard, Invitrogen™) was used for calibration.

*Transmission electron microscopy (*TEM). Drops of 5 µM β-Lg suspensions were deposited onto glow-discharged carbon-coated microscopy grids. The liquid in excess was blotted with filter paper and a drop of distilled water was deposited on the preparation in order to rinse out the residual glucose and buffer salts. The water in excess was blotted and, prior to drying, the preparations were negatively stained with 2% (w/v) uranyl acetate. The samples were observed using a JEOL JEM 1400 microscope operating at 120 kV. Images were recorded on camera Gatan Orius SC 1000 at Microscopy Rennes Imaging Center platform (MRic), situated in Rennes, France.

Dynamic light scattering. Particle size analysis was made on a Zetasizer nano ZS (Malvern Instruments, Orsay, France). Dynamic light scattering at a fixed angle of 173º was performed using a He-Ne laser, with λ = 633 nm. Samples were equilibrated at 20 ºC for 2 min and the characteristics of water was considered as solvent to the samples, the refractive index of water was 1.330, and the viscosity of water was 1.0031 mPa s at 20 ºC. This information was utilized in the computerization of the data to obtain the relaxation time

distribution, which was transformed into a distribution of hydrodynamic diameters, D_h, using Stokes-Einsten relationship: $D=kT/3\pi\eta D_h$, where k is Boltzmann's constant, T is the absolute temperature and η is the viscosity of water at 20 ºC.

Advanced glycation end products (AGE) fluorescence. According to the protocol described by Birlouez-Aragon et al. (1998) except that experiments were carried out in 96-microwell black plate format. Fluorescence measurements were performed with 200 µL samples on a Spectra Max M2 fluorescence spectrophotometer (Molecular Devices, Sunnyvale, California). Excitation and emission wavelengths were 330 and 420 nm, respectively. When necessary, samples were diluted in pH 7 phosphate buffer.

Size Exclusion Chromatography (SEC). The separation was achieved by SEC using a BioSep-SEC-S4000 column, size 300 x 7.8 mm (Phenomenex, Torrance, USA), on a Waters system. Elution was achieved with 0.05 M sodium phosphate buffer, pH 7, at 0.5 mL/min for 30 min, and detection of eluting proteins was performed at 214 nm. The standard proteins used for calibration were apoferritin (481.2 kDa); BSA (66 kDa); and α-lactalbumin (14.2 kDa).

RESULTS AND DISCUSSION

Heat-induced of β-lactoglobulin and β-casein

Heat-induced glycation of β-Lg and β-CN was monitored by LDS-PAGE technique (Figure 1). Compared with a control without glucose at the studied time, the presence of glucose slows down the aggregation process of β-Lg, as was already reported by our group (Pinto et al., 2012). This feature can be observed by the slower onset aggregates at the stacking gel to the glycated β-Lg gel. In contrast, for β-CN, aggregated species appeared faster when heating was performed in the presence of glucose. This protein associate mainly by hydrophobic bonds, which were dissociated in LDS-PAGE, however, in glycated β-CN samples, covalent bonds were produced, as described by Pellegrino et al. (1999). These authors showed large aggregates that progressively accumulated in the stacking gel when heating was performed in the presence of glucose. The occurrence of large amount of aggregates in the presence of glucose was confirmed by size exclusion chromatography (Figure 2B) which higher material

eluted at the void volume during the heating time, as compared to samples without glucose.

Pellegrino et al. (1999) also associated covalent aggregates in the glycated β-CN samples, with lysine-arginine crosslinking (pentosidine), however they inferred that other crosslink must therefore be considered, since the low pentosidine values found. The unknown nature of this reaction is related with the difficult to measure it.

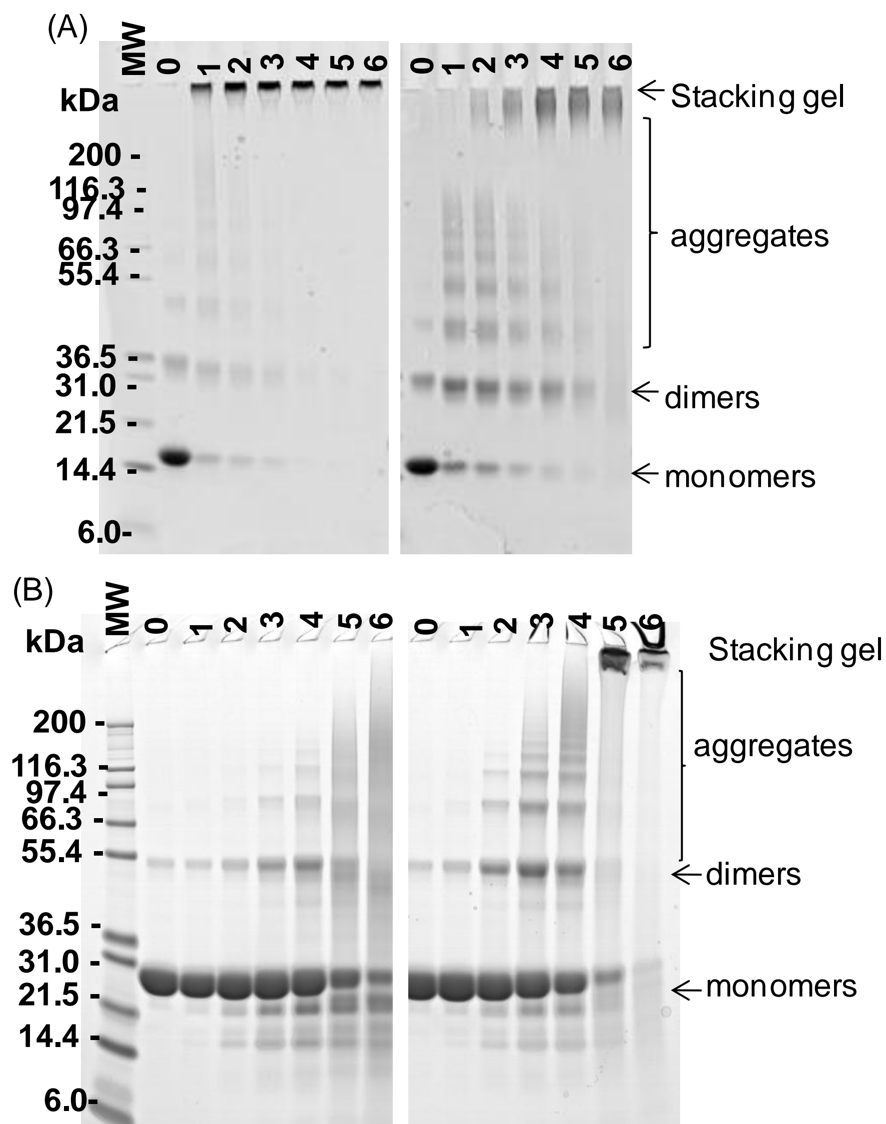

Figure 1: LDS-PAGE of β-lactoglobulin (A) and β-casein (B) without (left) or with (right) glucose heated 90 ºC/4 h. (0) native and heated protein at different times: (1) 10 min., (2) 45 min., (3) 2h, (4) 4h, (5) 12h and (6) 24h. MW = Molecular Weight Marker

Furthermore, in our work, the formation of covalent oligomers was also detected in no-glycated β-CN samples during heating time. These oligomers entered the separation gel but were not dissociated by LDS-PAGE. In SEC analysis, these oligomers/aggregates could be related to the slight increase (bulging to the left) of the base of native protein's peak, as has observed after 24 h-heating (Figure 2A).

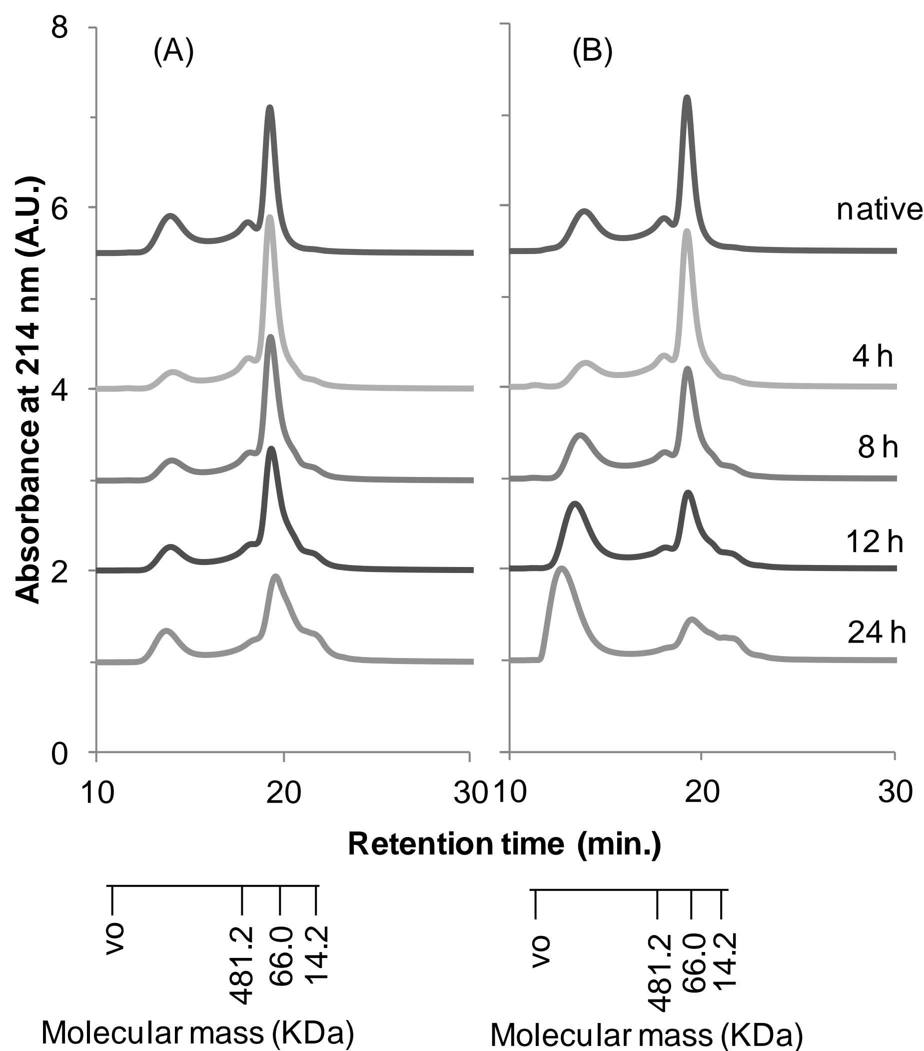

Figure 2 : SEC profiles of β-casein samples without (A) and with (B) glucose heating for 24 h. (Note:The secondary x-axis indicates the elution times of markers. vo=void volume (Column BioSep 14 – 200 Kda)

Figure 3 represent a schematic behavior of β-CN after heating with and without glucose. Before heating, β-CN exist as micellar form since the

concentration used was above the micellar concentration of β-CN (Kull et al., 1997). Morerover, Ossowski et al. (2012) fitted recently, a polydisperse ellipsoid core model to β-CN samples analyzed by small-angle neutron scattering. β-CN monomers associate via hydrophobic bonding into micelles (O'Connell et al., 2003). The protein micelles (Figure 3A) with a average hydrodynamic diameter of 28 nm were completely dissociated under LSD presence (Figure 3B).

When heated, covalent interactions were favored between molecules inside the micelle (Figure 3C). However, these heat-induced covalent aggregates was not sensible to LDS (Figure 3D). Furthermore, according to Andrews et al. (1979) these thermal-aggregate are spherical with a radius of about 100 Å. The interior of the aggregate is relatively disordered, and the β-CN molecules remain in a largely flexible, hydrated conformation.

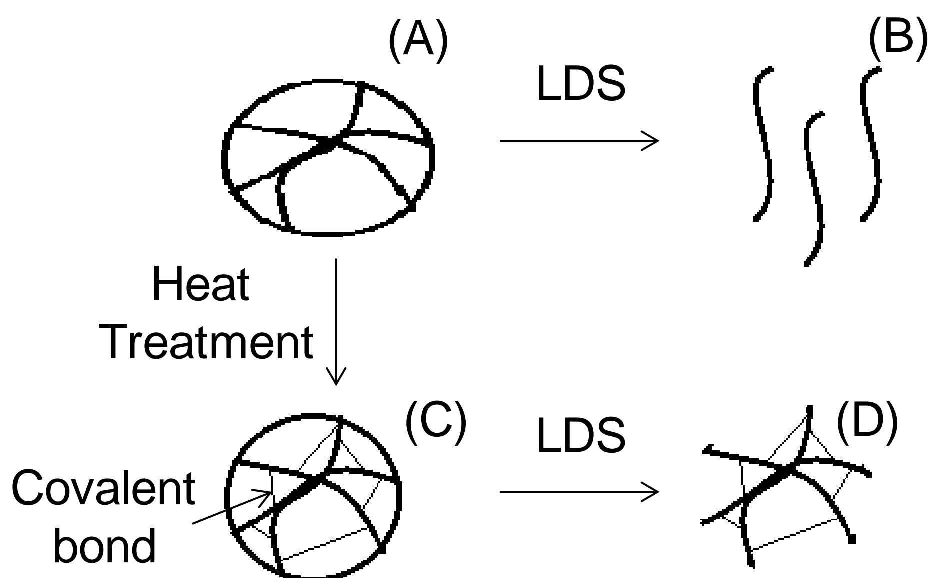

Figure 3: Schematic representation of β-casein behavior heated 90 ºC in a concentration about 0.5 g/L. Native β-casein (b) heated β-casein; (c) native β-casein reduced with LDS; (d) heated β-casein reduced with LDS; LDS = lithium dodecyl sodium

Transmission electron microscopy (TEM) was used to observe the morphology of heat-induced aggregates. β-CN showed *clustered* small

spherical particles that differ from the morphology found for β-Lg aggregates, with the presence of a mixture of aggregates and “small fibrils” (Figure 4), which was also recently observed by Ossowski et al. (2012). We can also observe, by TEM images, the differences between glycated and no-glycated samples. Spherical particles of about 10-20 nm were found for aggregates from β-CN heated 90 ºC for 24 h. Besides, 24 h-glycated β-CN maintained the spherical shape but presented a greater diameter (20 – 50 nm). The presence of aggregated species in the image of β-CN samples is probably due to the effect of negative staining, i.e, uranyl ions can bind to proteins, even if it should not bind to the specimen.

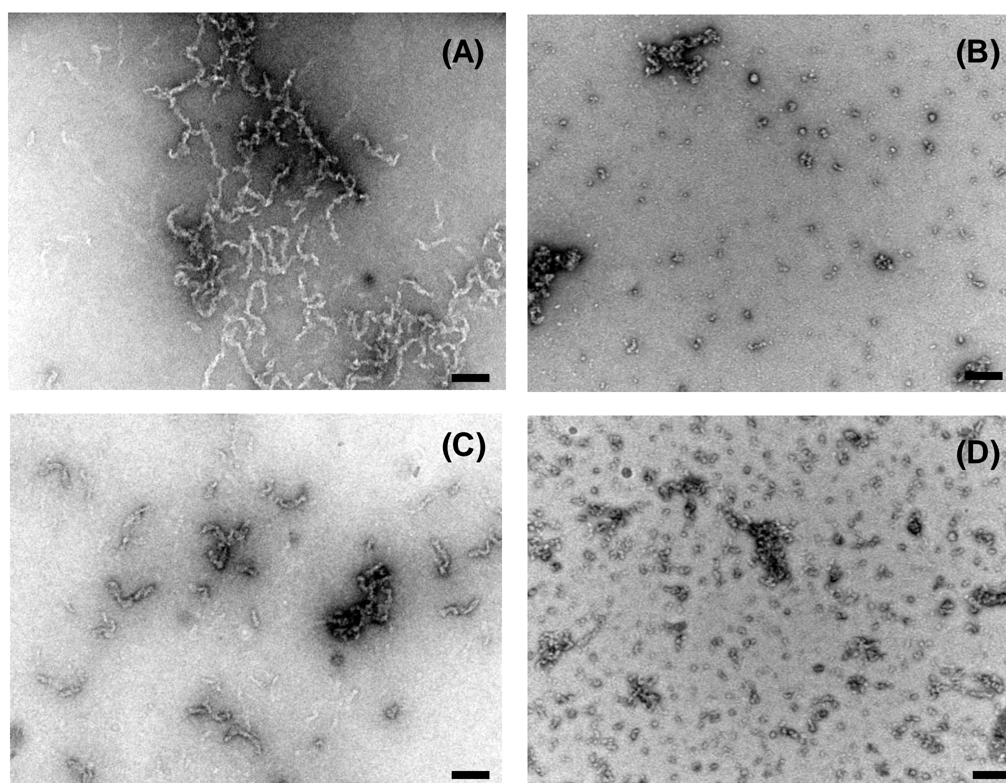

Figure 4: TEM images of heated 90 ºC for 4h (left) or 24h (right) β-lactoglobulin (left) and β-casein (right) without (A and B) or with (C and D) glucose. Bars=100 nm

There are some divergences between these data and hydrodynamic size data (Table 1). In the last, the difference between the β-CN glycosilated or no, was not observed, which can be related with the heterogenic size distribution to the glycated sample (data no shown). When DLS sizing data is compared to

Transmission Electron Microscopy images, the aggregation state of the particles can be determined. In an unagglomerated suspension, the DLS measured diameter will be similar or slightly larger than the TEM size, since one of the steps of sample preparation in TEM method can promote dehydration of protein.

In no-glycated systems, the observed width of the β-Lg fibrils was about 9 nm while the fibrils formed with glucose exhibited larger width of about 18 nm. The TEM images of the β-Lg sample heated with glucose showed a mixture of fibrils (about 50 to 200 nm in length) and aggregates. Twisted fibrils separated or aggregated were observed when β-Lg was heated without glucose.

Dynamic light scattering analysis (Table 1) showed an increase of the aggregates from around 7 to 79 nm and from 7 to 63 nm, to β-Lg without or with glucose, respectively, in 2h of heating. Furthermore, this data confirmed our previous results that native β-Lg was not aggregate and presence of glucose slows down the aggregation process of β-Lg (Pinto et al., 2012), since hydrodynamic size of the samples increased, however the values of glycated samples at 24h heating were higher than no glycated one.

Table 1: Hydrodynamic size (nm) of the native and 90 ºC/4 h heated β-casein and β-lactoglobulin with or without glucose

	size (nm)		
heating time (h)	0	2	24
β-lactoglobulin	6.96	79.17	90.05
β-lactoglobulin + glucose	6.96	63.13	67.83
β-casein	28.0	28.5	35.1
β-casein + glucose	28.0	29.1	31.6

As we already performed for β-Lactoglobulim under the same experimental conditions (paper 1, Pinto et al., 2012), the glycation of β-CN was monitored by fluorescence intensity of advanced end product (AGE) according to Birlouez-Aragon et al. (1998). Figure 5 shows the evolution of AGE after heating of β-CN at 90°C over 24 h with and without glucose. Fluorescence intensitiy values of 168 and 940 were covered after 4h heating without and with glucose, respectively.

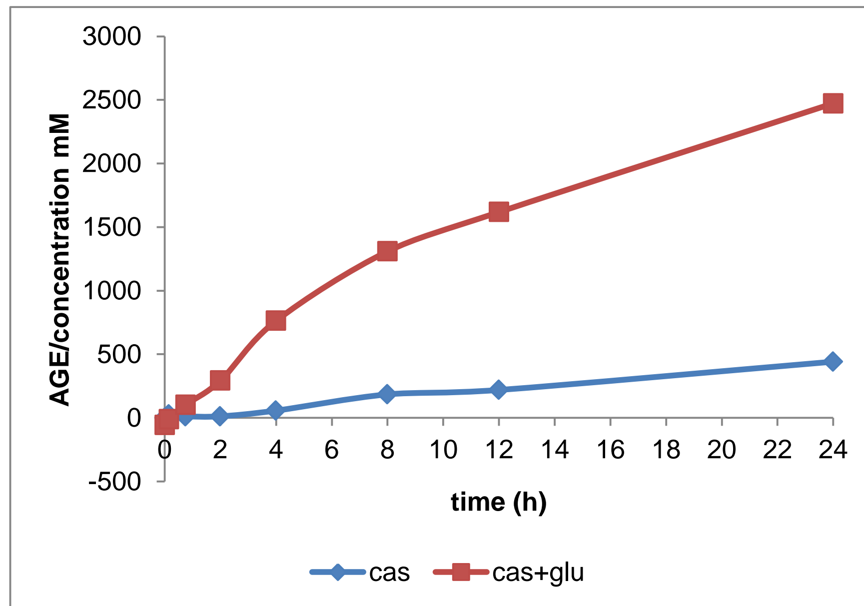

Figure 5: AGE fluorescence of the native and heated (90°C for 24 h) β-casein with or without glucose. AGE was expressed per mmole of protein.

In vitro digestion of heated proteins- Native and heated proteins (90 ºC for 2 and 24 h) with or without glucose were subjected to *in vitro* digestion. The LDS-PAGE profile of the native proteins digested by gastric and duodenal enzymes were presented in Figure 6 and these results are in accordance with published works (Mandalari et al., 2009; Dupont et al., 2010a; Barbé et al., 2013). Native β-Lg resists to 90 min-gastric/duodenal digestion and was not significantly altered. In contrast, native β-CN was rapidly digested and completely disappears after 20 min incubation with pepsin solution.

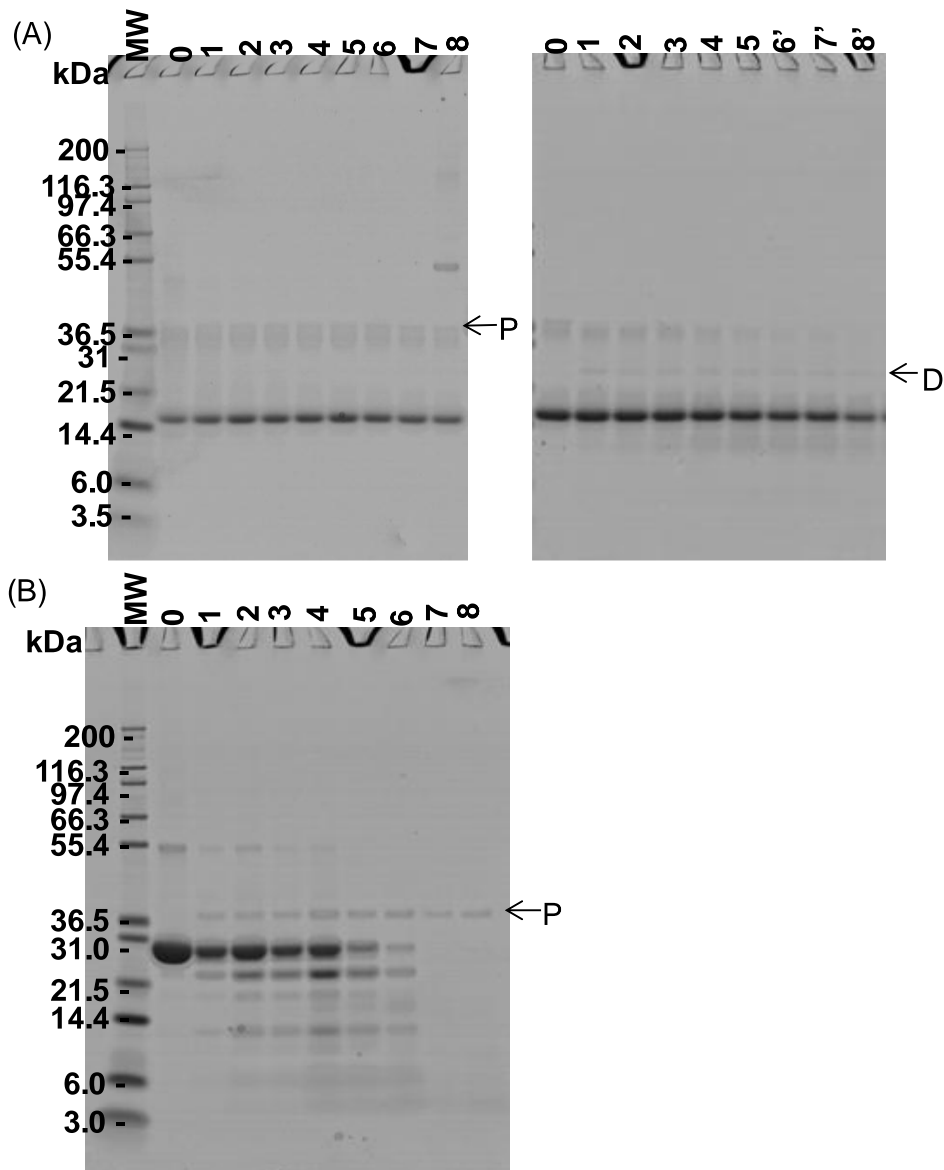

Figure 6: LDS-PAGE of native (A) β-lactoglobulin and (B) β-casein digested gastric (left) and duodenal (right); profile without enzyme (0), or with enzyme for (1) 0min (2) 1 min, (3) 2min, (4) 5 min, (5) 10 min, (6') 15 min, (6 and 7') 20 min; (8') 30 min, (7) 40 min; (8) 60 min. MW = Molecular Weight Marker. Arrows: P(Pepsin); D(Duodenal enzymes)

When proteins are heated, the digestion profiles changed. As was expected, and already demonstrated by others (Mandalari et al., 2009; Barbé et al., 2012) heat denatured β-Lg become sensitive to digestion and was completely digested after 40 min incubation under gastric conditions. Heated β-CN also appeared to be slightly more sensitive to pepsin compared to unheated since β-CN 5 min were enough to digest formed aggregates. In the presence of

sugar, the analysis conducted by LDS-PAGE showed that glycated samples seemed to be more resistant than no glycated one (Figure 7 and 8). Aggregated forms of β-CN were still detected after 40 min incubation with pepsin. Interestingly, LDS-PAGE shows the progressive digestion of large aggregates (Figure 8, line 2) to small ones that enter the gel (see Figure 8, line 6). Nearly, complete digestion to peptides was achieved before adding duodenal enzymes, with and without glucose. Heating in the presence of glucose induced more drastic change in the case of β-Lg. As shown in Figure 7B, aggregates formed in the presence of glucose (top of the gel) are still present at the end of gastro-intestinal digestion process. Without glucose, 40 min incubation under gastric conditions was sufficient for total digestion of aggregates from heated β-Lg.

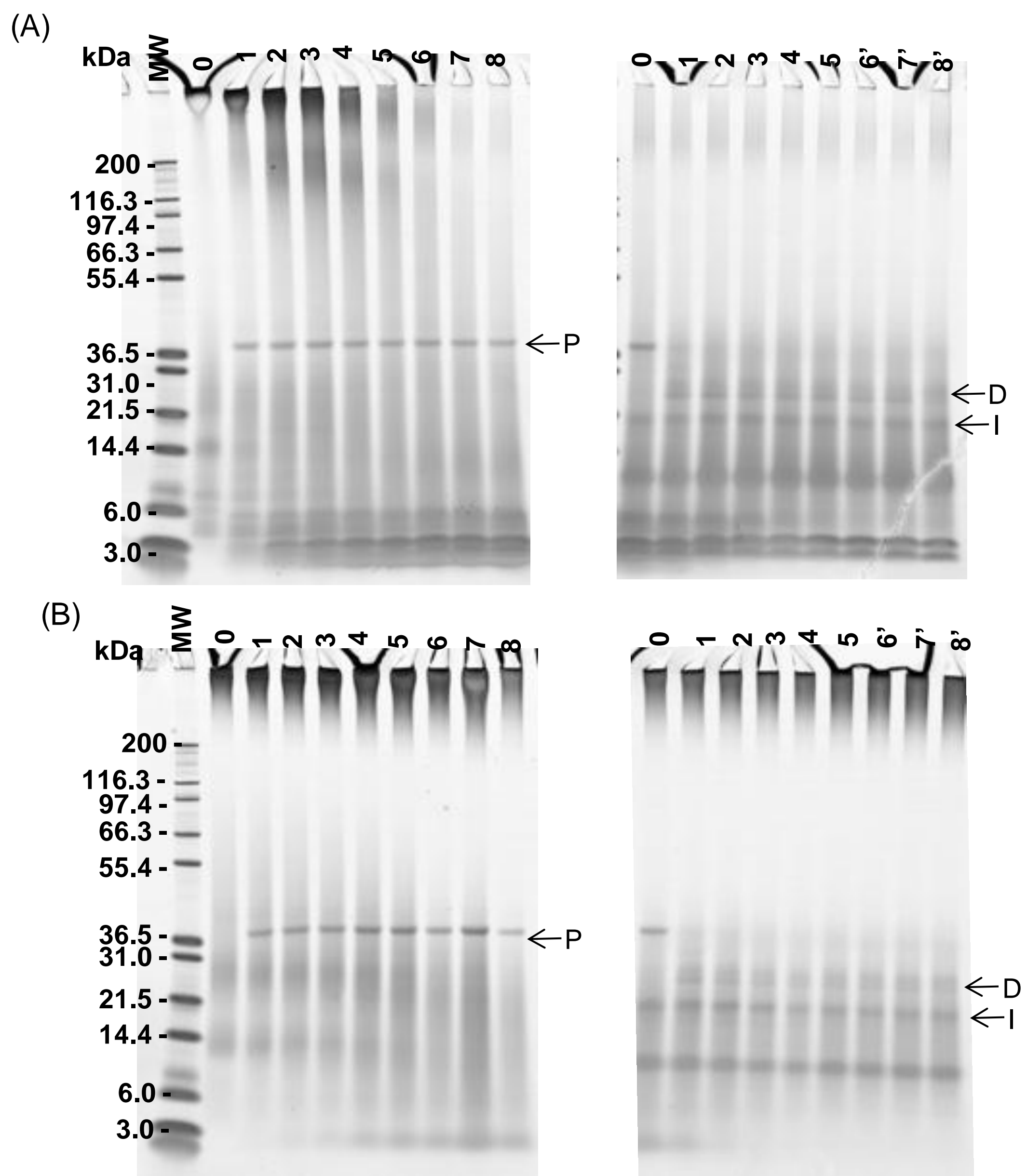

Figure 7: LDS-PAGE of 90 ºC/24h heated (A) β-lactoglobulin and (B) β-lactoglobulin + glucose digested gastric (left) and duodenal (right). Profile without enzyme (0), or with enzyme for (1) 0min, (2) 1 min, (3) 2min, (4) 5 min, (5) 10 min, (6') 15 min, (6 and 7') 20 min; (8') 30 min, (7) 40 min; (8) 60 min. MW = Molecular Weight Marker. Arrows: P (Pepsin); D (Duodenal enzymes); I (inhibiteur duodenal enzymes)

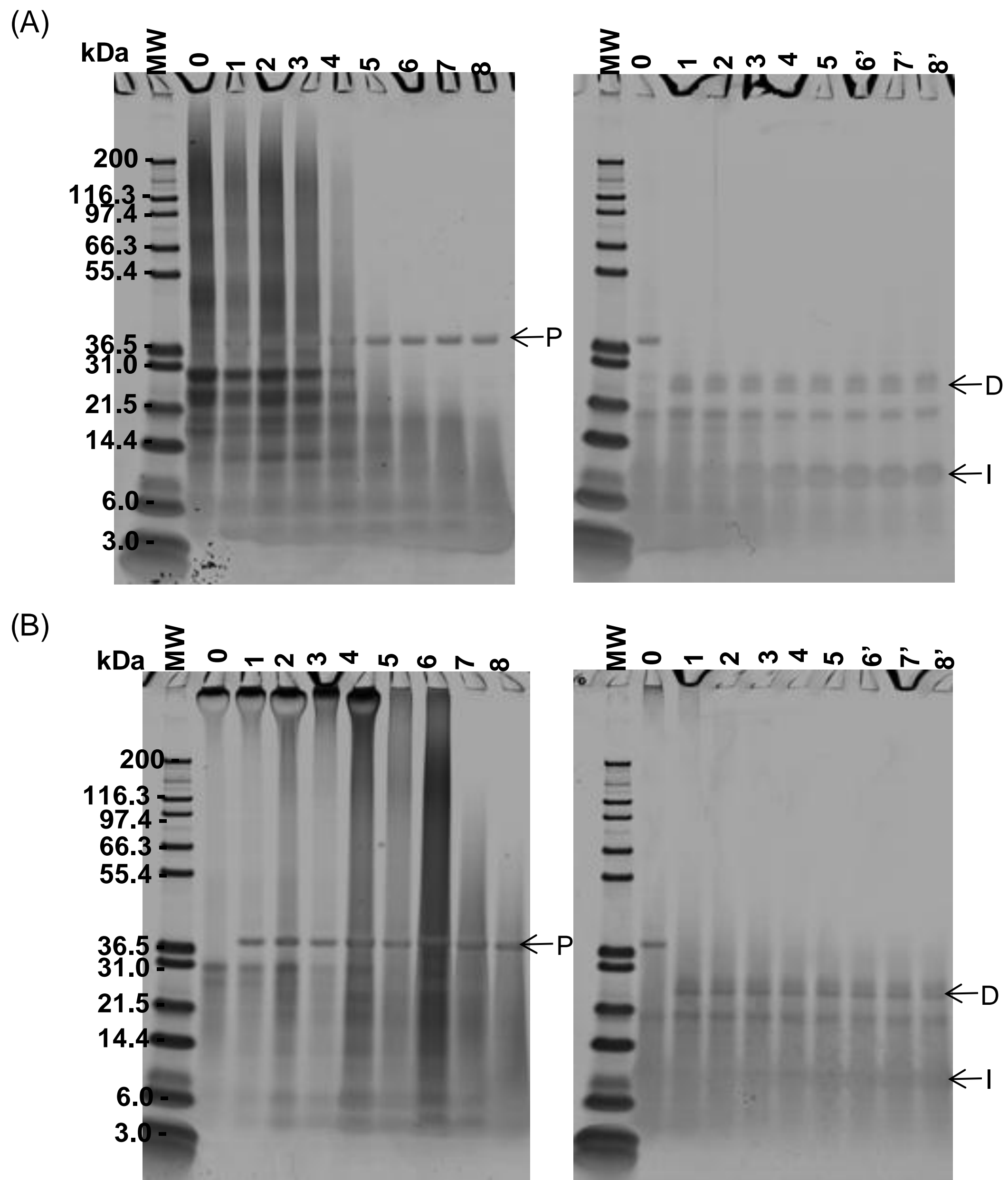

Figure 8: LDS-PAGE of native (A) and 90 ºC/24h heated β-casein without (B) or with (C) glucose digested gastric (left) and duodenal (right). Profile without enzyme (0), or with enzyme for (1) 0min (2) 1 min, (3) 2min, (4) 5 min, (5) 10 min, (6') 15 min, (6 and 7') 20 min; (8') 30 min, (7) 40 min; (8) 60 min. MW = Molecular Weight Marker. Arrows: P(Pepsin); D(Duodenal enzymes); I (inhibiteur duodenal enzymes)

These results confirm those of Corzo-Martinez et al. (2010) who showed that glycation with galactose or tagatose impaired β-Lg proteolysis and immunoreactivity. They suggest that protein aggregation induced in the presence of sugars may protect the protein during *in vitro* proteolysis. In

contrast, glycation has also been shown to increase protein proteolysis due to conformational changes produced during glycation (Bouhallab, et al.; 1999; Yeboah et al., 2004). This apparent discrepancy may be attributed to the progress of Maillard reaction in various studies, early versus very advanced, that control the aggregation state of the proteins.

The resistance of glycated β-Lg to the digestion was also observed in TEM images (Figure 9). Heated β-Lg was described as twisted fibrils, which in some cases have been described as a form of aggregation that are stable and resistant to degradation by proteases (Ecroyd et al., 2008). However, in this work, we can observe that β-Lg twisted fibrils were broken down into small particles. Digestion of sample heated in the presence of glucose showed different picture. Small as well as large particles were detected.

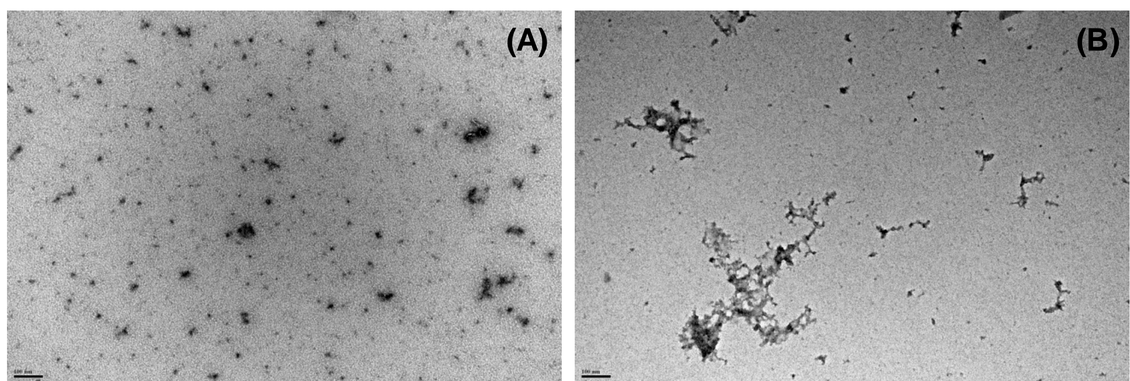

Figure 9: TEM images of heated 90 ºC/4 h β-lactoglobulin (A) and β-lactoglobulin+glucose (B) and subjected to gastric/duodenal digestion. Bars=100 nm

CONCLUSION

The presence of glucose highly impacts the heat-induced aggregation behavior of studied milk proteins, i.e. β-Lg, the well-structured globular one and β-CN, the unstructured protein. Aggregates produced in the presence of glucose, in particular in the case of β-Lg, were shown to be more resistant to simulated gastro-intestinal digestion. Even if severe heating was applied in the present study (90°C, 24h), the occurrence of such type of resistant aggregates in formulated food is not excluded. The biological properties and consequences deserve to be evaluated in a future work.

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4. β-LACTOGLOBULIN, κ-CASEIN AND α_{S2}-CASEIN: HEAT-INDUCED AGGREGATION

When milk is heated, numerous reactions and interactions take place between milk components leading to formation of numerous aggregates for which the well- known is the mixed protein aggregates formed between κ-casein (κ- CN) arising from casein micelle and β-lactoglobulin (β-Lg), the major protein of whey. This system has extensively been studied because it is very important to understand synergistic effect that can occur between protein during heat treatment and consequently better control heat milk processing. The interaction between κ-CN and β-Lg mainly intervenes by disulfide exchange between the two proteins. However other proteins are susceptible to play the same role that κ-CN and formed such as complex. α_{S2}-CN has 2 cystein residues and it is susceptible to establish interchange disulfide bond (Rasmussen et al, 1999). In addition α_{S2}-CN occurs at the same concentration and the presence at the casein micelle surface has been established.

Milk system is highly complex and hence models systems remain extremely important to gain information on the events occurring *in situ* in milk during processing. There is little in the literature in the interaction between mixed protein systems due to difficulties to evidence these complexes as stated in bibliographic section and often heated aggregation process give rise to a numerous aggregates for each partner protein and for the mixed systems. This part of the work was a preliminary study to explore this complex system.

Our objective was for article 3 to explore and identify the possible interaction between β-Lg and α_{S2}-CN in investigating the formation of such a complex. The same approach was made for the system β -Lg and κ-CN.

PAPER 3: Heat treatment influences the aggregation of β-lactoglobulin/α_{S2}-casein and β-lactoglobulin/κ-casein and their *in vitro* digestion

Heat treatment influences the aggregation of β lactoglobulin/α_{S2}-casein and β- lactoglobulin/κ-casein and their *in vitro* digestion

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ABSTRACT

Heat treatment is commonly used in the field of dairy technology. In some cases, milk is heated/cooled many times before to obtain the final product and milk components are subject to the combined effects of heat treatment. The aim of this work was to study the effects of a sever heat treatment (90 ºC/2 h) on purified milk proteins (β-lactoglobulin, α_{S2}-casein and κ-casein) and the consequences on the *in vitro* digestion of the formed aggregates. These proteins were heated in simple or binary systems. To obtain this information, before and/or after digestion the following techniques were used: gel electrophoresis (LDS-PAGE), transmission electron microscopy, size exclusion chromatography, fluorescence and dynamic light scattering. Mixed systems seem more reactive because the aggregates were formed faster than when these proteins were heated separately. Moreover, high temperatures have favored the formation of covalent bonds including disulfide bridges. Furthermore, some changes during the *in vitro* digestion of these aggregates proteins have been evidenced.

O tratamento térmico influencia a agregação de β-lactoglobulina/α_{S2}-caseína e β-lactoglobulina/κ-caseína e sua digestão *in vitro*

RESUMO

O tratamento térmico é normalmente usado em tecnologia de laticínios. Em alguns casos o leite é aquecido/resfriado várias vezes antes de se obter o produto final. Desta forma, os componentes do leite estão sujeitos aos efeitos sucessivos do tratamento térmico. O objetivo deste trabalho foi estudar os efeitos de um tratamento térmico severo (90 ºC/2h) sobre proteínas do leite purificadas (β-Lg, α_{S2}-casein and κ-casein) e as consequências da digestão *in vitro* dos agregados formados. Para obter essas informações antes e depois da digestão *in vitro*, as seguintes técnicas foram utilizadas: LDS-PAGE, microscopia eletrônica de transmissão, cromatografia de exclusão molecular, fluorescência e difusão dinâmica da luz. Os sistemas mistos parecem ser mais reativos, pois os agregados são formados mais rápidos do que nos sistemas em que as proteínas são aquecidas separadamente. O tratamento térmico intenso favoreceu a formação de ligações covalentes e pontes disulfeto. Além disso, algumas mudanças durante a digestão *in vitro* dos agregados foram observadas.

Le traitement thermique influence l'agrégation et la digestion *in vitro* des mélanges β-lactoglobuline/ caséine-α_{S2} et β-lactoglobuline/caséine-κ

RESUME

Le traitement thermique est communément utilisé dans le domaine de la technologie laitière. Dans certains cas, le lait est chauffé/refroidi de nombreuses fois avant d'obtenir le produit final et les composants du lait sont soumis aux effets cumulés du traitement thermique. Le but de ce travail était d'étudier les effets d'un traitement thermique intense (90 ºC/2 h) sur les protéines de lait purifiées (β-lactoglobuline Lg, caseines $\alpha_{S2\ et}$ κ) ainsi que ses conséquences sur la digestion *in vitro* des agrégats produits. Afin d'obtenir ces informations, avant et/ou après digestion les techniques suivantes ont été utilisées : l'électrophorèse sur gel (LDS-PAGE), la microscopie électronique à transmission, la chromatographie d'exclusion moléculaire, la fluorescence et la diffusion dynamique de la lumière. Les systèmes mixtes semblent plus réactifs du fait que les agrégats étaient formés plus rapidement que lorsque ces protéines étaient chauffées séparément. De plus, les hautes températures ont favorisé la formation des liaisons covalentes incluant des ponts disulfure.

INTRODUCTION

Milk is a complete food that contains fat, proteins, carbohydrates, minerals, vitamins, enzymes and a lot of minor components, whose biological role is the nutrition of the young. To be commercialized and transformed, cow milk and dairy products, are normally heated. In some cases, milk can be heated several times before to obtain final product. Heat treatment can promote damages in milk constituents, such as important changes in proteins.

Cow milk contains approximately 3.3 % of proteins, of which 80 % are caseins that are the protein precipitated from milk at pH 4.6. The remainder is mainly whey proteins (Fox and McSweeney, 2003; Walstra, 2006). β-lactoglobulin (β-Lg) is the major whey protein in bovine milk, is a single polypeptide chain of 162 amino acids with a molar mass of 18.3 kDa. In neutral pH it exists in the dimeric form. Its tertiary structure is composed of nine anti-parallel β-sheets and one α-helix, which encloses a hydrophobic pocket as internal binding site and β-Lg contain two intramolecular disulfide bonds and one free sulfhydryl group (Fox and McSweeney, 2003; Kontopidis et al., 2004). The mechanism of heat aggregation of β-Lg is relatively well-established (De Wit, 2009). Upon heating at about 70°C, the dimers dissociate into monomers which partially unfold before to form aggregates via disulphide and non-covalent interactions. This subject has been reviewed by some groups, such as Angel de la Fuente et al. (2002), Kontopidis et al. (2004), de Wit (2009) and Nicolai et al. (2011).

In the milk system, heat treatment promotes dissociation of different caseins, mainly κ-casein (κ-CN), from the micelles to the soluble phase that reacts with β-lactoglobulin to form a complex (Anema and Li, 2003). Complex formation between β-Lg and κ-CN has been studied in pure protein model systems close to the system milk to understand the pathway of this aggregation that involves a sulphydryl-disulphide interchange mechanism. K-CN is a single polypeptide chain of 169 amino acid residues with a molar mass of 19 kDa. Two cysteine residues give the possibility of disulfide bond formation (Farrell et al., 2004).

However in milk several other proteins with possibility of establish this kind of interaction exists. In the case of caseins, α_{S2}-casein (α_{S2}-CN) is a

polypeptide with 207 amino acids including 2 cysteine residues and a molar mass of 25.2 kDa, in a unorganized structure. The presence of cysteine allows, inter or intramolecular, thiol-disulfide interchange reactions, furthermore, due to its nature amphiphilic self-association is possible from forces of attraction between the positively charged domains of a molecule with those of another negatively charged molecule (Sgarbieri, 1995). α_{S2}-CN is present primarily as a monomer with an internal disulfide but dimers with the chain either parallel or antiparallel to each other has been reported as well as possibility of polymerization with superior order (Rasmussen and Petersen, 1991).This prone us to investigate the possible interaction between β-Lg and α_{S2}-CN by investigating the complex formed upon heat treatment.

Heat treatment and formation of milk protein aggregates could be responsible for changes in proteolysis (Dupont et al., 2010a). The formation of heated aggregates may be resistant to digestion and increase the allergenic potential of milk (Mantyjarvi et al., 2000; Wal, 2001; Dupont et al., 2010a).

The aim of this work was to investigate the heat-incuced aggregation process of a mixture of β-Lg and κ-CN and β-Lg and α_{S2}-CN and to evaluate the sensitivity of formed aggregates to *in vitro* digestion. Aggregation and digestion of individual proteins were used as references.

MATERIAL AND METHODS

Materials. All the chemicals and standards used were obtained from Sigma. Fresh bovine raw milk from homozygous cows was obtained from the experimental dairy farm (INRA, Rennes, France). κ-CN was prepared by selective precipitations (Zittle and Custer, 1963) and fractionated according to the method of Guillou et al. (1897), modified by Leonil et al. (2008). β-LG A was prepared from the milk of homozygous cows as reported by Leonil et al. (1997), in which membrane processes and low temperatures (below 56 °C) are involved. The freeze-dried β-LG A have a purity of 98% as determined by reversed-phase HPLC analysis. Protein concentration was determined by measuring the absorbance at 280 nm, using extinction coefficient values of 0.930 and 0.937 g/L/cm for κ-CN and β-Lg, respectively.

Heat treatment. Solutions with equimolar concentrations were prepared in sodium phosphate buffer 0.1 M, pH 7, containing 0.30 mM. Model system

solutions were heated at 90 °C for 2 h in eppendorf tubes. After predetermined times between 0 and 2 h, the tubes were removed and cooled in an ice-water bath. Each sample was made in duplicate.

Enzymatic digestion *of* aggregates. We followed the method described by Dupont et al. (2010b) with modifications. Samples of β-Lg, α_{S2}-CN and κ-CN or the mixtures native and heated 90ºC/2h, was dissolved in simulated gastric fluid (1mg/mL), Porcine gastric mucosa pepsin (activity 3.802 U/mg of protein) was added to give 182 U of pepsin/mg of β-Lg, α_{S2}-CN and κ-CN (0.1 mM, final concentration). Aliquots (100 µL) were removed over the 60 min. digestion time course. Pepsinolysis was stopped by raising the pH using 0.5 M sodium bicarbonate. Before duodenal proteolysis, the pH of samples was adjusted to 6.5 by addition of 0.1M NaOH and duodenal digestion components added to give final concentrations as follows: 4 mM sodium taurocholate, 4 mM sodium glycodeoxycholate, 26.1 mM bis-Tris buffer pH 6.5, 0.4 U/mg of β-Lg and κ-CN bovine. α-chymotrypsin (activity 52.6 U/mg of protein), 34.5 U/mg of β-Lg or κ-CN porcine trypsin (activity 13.390 U/mg of protein). Aliquots (100 µL) were removed over the 30 min digestion time course, and proteolysis stopped by addition of a twofold excess of soybean Bowmann-Birk trypsin-chymotrypsin inhibitor above that calculated to inhibit trypsin and chymotrypsin in the digestion mix.

Characterization of aggregates and digested proteins

Gel electrophoresis. The samples were analyzed by polyacrylamide gel electrophoresis (PAGE) using NuPage® Novex® (4-12%, Bis-Tris Mini Gels 1.5 mm, Invitrogen™) under reducing and non-reducing conditions. Disulfide bonds were reduced by an overnight incubation in the NuPage® Lithium Dodecyl Sulphate (LDS) sample buffer containing 30 mM Dithiothreitol (DTT) at room temperature. Samples containing approximately 12 µg of protein were analyzed. Gels were stained with Coomassie Brilliant Blue R250. A high molecular weight markers kit (3-200 kDa, Mark 12™ Unstained Standard, Invitrogen™) was used for calibration.

*Transmission electron microscopy (*TEM). Drops of 5 µM of proteins suspensions were deposited onto glow-discharged carbon-coated microscopy grids. The liquid in excess was blotted with filter paper and a drop of distilled

water was deposited on the preparation in order to rinse out the residual glucose and buffer salts. The water in excess was blotted and, prior to drying, the preparations were negatively stained with 2% (w/v) uranyl acetate. The samples were observed using a JEOL JEM 1400 microscope operating at 120 kV. Images were recorded on camera Gatan Orius SC 1000 at Microscopy Rennes Imaging Center platform (MRic), situated in Rennes, France.

Thioflavin T assay. Thioflavin T (ThT) can bind the amyloid fibrils by ionic and hydrophobic interactions changing the fluorescence excitation spectrum. An increase in ThT fluorescence intensity is related to a ThT bound along the length of the amyloid fibrils (Khurana et al., 2005). To perform the ThT fluorescence measurement, a ThT stock solution (0.63 mM) was prepared in phosphate buffer at pH 7 and stored at 4°C.

To verify if fibrils were formed, experiments were carried out into a four-sided quartz cuvette: a working solution was prepared by diluting the stock solution in phosphate buffer and added to the protein solutions so that the final dye and protein concentrations were 0.58 and 0.023 mM, respectively. Fluorescence measurements were performed on a Spectra Max M2 (Molecular Devices, Sunnyvale, California) fluorescence spectrophotometer at an excitation wavelength of 446 nm and an emission wavelength of 478 nm (475 nm cutoff). The net intensities were obtained after subtraction of the background signal. Results are expressed as mean of five independent experiments.

Dynamic light scattering. Particle size analysis was made on a Zetasizer nano ZS (Malvern Instruments, Orsay, France). Dynamic light scattering at a fixed angle of 173º was performed using a He-Ne laser, with λ = 633 nm. Samples were equilibrated at 20 ºC during 2 min. The refractive index value of 1.330 and the viscosity value of 1.0031 mPa s were used for water at at 20 ºC. These data were usedd in the computerization of the data to obtain the relaxation time distribution, which was transformed into a distribution of hydrodynamic diameters, D_h, using Stokes-Einsten relationship: $D=kT/3\pi\eta D_h$, where k is Boltzmann's constant, T is the absolute temperature and η is the viscosity of water at 20 ºC.

Size Exclusion Chromatography (SEC). The separation was achieved by SEC using a BioSep-SEC-S4000 column, size 300 x 7.8 mm (Phenomenex,

Torrance, USA), on a Waters system. Elution was achieved with 0.05 M sodium phosphate buffer, pH 7, at 0.5 mL/min for 30 min, and detection of eluting proteins was performed at 214 nm. The standard proteins used for calibration were apoferritin (481.2 kDa); BSA (66 kDa); and α-lactalbumin (14.2 kDa).

Degree of hydrolyse. The proportion of cleaved peptide bonds in protein hydrolysates was measured by chemical reaction with trinitrobenzenesulfonic acid (TNBS), which reacts with amino acids, yielding a yellow product (absorbance at 340 nm). The method used was described by Adler-Niessen (1979) with modifications. The TNBS Sigma reagent was diluted in water to obtain 0.1% (w/v). Experiments were carried out in 96-microwell plate format (Costar), with 200 µL of sample per plate on a Spectra Max M2 (Molecular Devices, Sunnyvale, California) spectrophotometer. Triplicate aliquots (10 µL) of test or standard solutions were added to 100 µL borate potassium buffer (1 M, pH 9.2) and 40 of diluted TNBS followed by mixing and incubation at 37°C for 60 min in a incubator. Samples were protected to light during all reaction. After incubation, the reaction was stopped by lowering the pH to neutrality with the addition of 40 µL of a solution of monosodium phosphate (2 M) and sodium sulfite (18 mM) in each well. Finally, absorbance values were measured and the content of NH_2 was calculated with a standard curve of glycine.

RESULTS

Nonreducing and reducing PAGE was performed to characterize protein aggregation as a function of heating time. Figure 1, shows LDSPAGE of heated α_{S2}-CN, β-Lg and the mixture of both at 90°C. α_{S2}-CN has a high propensity to polymerize in solution (Rasmussen et al, 1999). We observed that this protein presented a heterogeneous profile with numerous bands for which a dimer at about 36.5 kDa, which was observed by Rasmussen et al. (1999). This electrophoretic profile was weakly modified during heat treatment (Figure 1A). Decrease of dimer bands was observed after 45 min concomitantly to the increase of aggregates bands up to 200 kDa. α_{S2}-CN monomers bands apparently have not changed, owing to their inherent structural disorder.

The reduced gel with DTT indicates that some of these α_{S2}-agregates, and dimers were linked by disulfide bonds, as showed by Hoagland et al.

(2001), even if significant part of the oligomers, in particular dimers resisted to DTT, at the experimental conditions: 0.02 M of DTT, room temperature for 18h. No material was detected in the stacking gel. Figure 1B shows that β-Lg native exists in monomer and dimer forms, which have aggregated during heating time. Several bands showed a great variety of aggregates, which may be associated each other, since the smaller bands decreased with increase of the bands in the stacking gel. These aggregates produced during 2 h of heating were linked mainly by disulfide bonds, which were reduced in presence of DTT (Figure 1B, right). This was in accordance with our previous study (Pinto et al., 2012). Finally, when α_{S2}-CN was heated with β-Lg we observed, the same behavior described above for each protein separately, with some changes i- a decrease of the band of α_{S2}-CN dimer was more pronounced and there were less aggregates up to 200 kDa, conversely β-Lg monomers band disappeared faster when heated in presence to α_{S2}-CN; ii- the protein mixture presented large aggregates in the stacking gel, like when β-Lg was heated alone; however, when reduced with DTT, mixed of aggregates remained in the stacking gel, iii- bands like trimers of the β-Lg (about 51 kDa) was more evident when the heated mixture was reduced with DTT. Besides β-Lg trimers, these aggregates could be hetero-aggregate formed by monomer of β-Lg (~ 18 kDa) with dimer of α_{S2}-CN (~ 34 kDa).

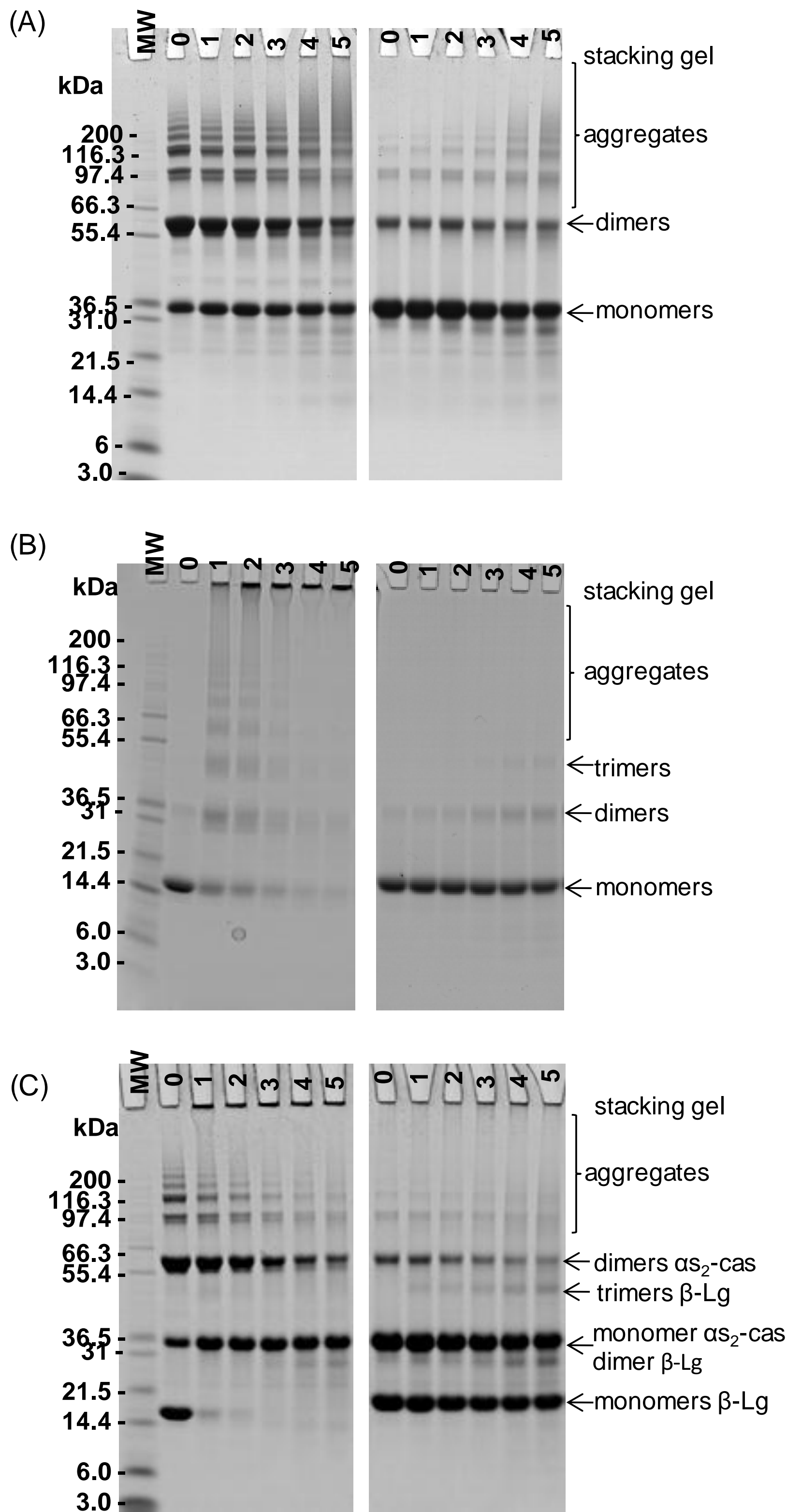

Figure 1: LDS-PAGE of α_{S2}-casein (A), β-lactoglobulin (B) and (C) α_{S2}-casein + β-lactoglobulin, heated 90 ºC/2h, non reduced (left) and reduced (right) with DTT. (0) Native protein and (1) 10 min, (2) 20 min, (3) 45 min, (4) 90 min and (5) 2h of heating. MW = Molecular Weight Marker

The heat induced aggregation at 90°C, 2h of individual or mixed κ-CN and β-Lg as followed by LDS-Page is shown in Figure 2.

Native κ-CN (Figure 2A) presented a mixture of monomers, dimers and aggregates species, which is related with its property to self-association (Farrell et al., 1996). After 10 min of heating, κ-CN sample was mainly composed by large aggregates stopped in stacking gel, which has been reported by Groves et al. (1998). The monomers bands decreased with the heating time.

When β-Lg was heated with κ-CN (Figure 2B), some differences in the gel profile were observed compared with the gels of proteins heated separately, as will be explained after. Furthermore, a band around 50 kDa, already present in native κ-CN dimers, was observed after heating both proteins together (Figure 2B, left). Interestingly, this band was present whatever the heating time of mixed proteins while it disappeared in favor of large aggregates immediately after heating κ-CN alone. The composition of this dimeric band differs probably between the two samples κ-CN alone or in mixture with β-Lg. Finally, in the no reduced gel (Figure 2, left), all samples have presented aggregates in stacking gel.

When these samples were reduced with DTT, materials in the stacking gel were detected only in the gel of κ-CN heated alone (Figure 2A, right). In the other sample, large aggregates were completely reduced in species that entered into the gel (Figures 2B, right). In the reduced gel of the mixed proteins, the band corresponding to κ-CN monomer or β-Lg dimer was recovered in all heated samples. In contrast, only part of κ-CN monomers was recovered when the protein was heated alone above 45 minutes.

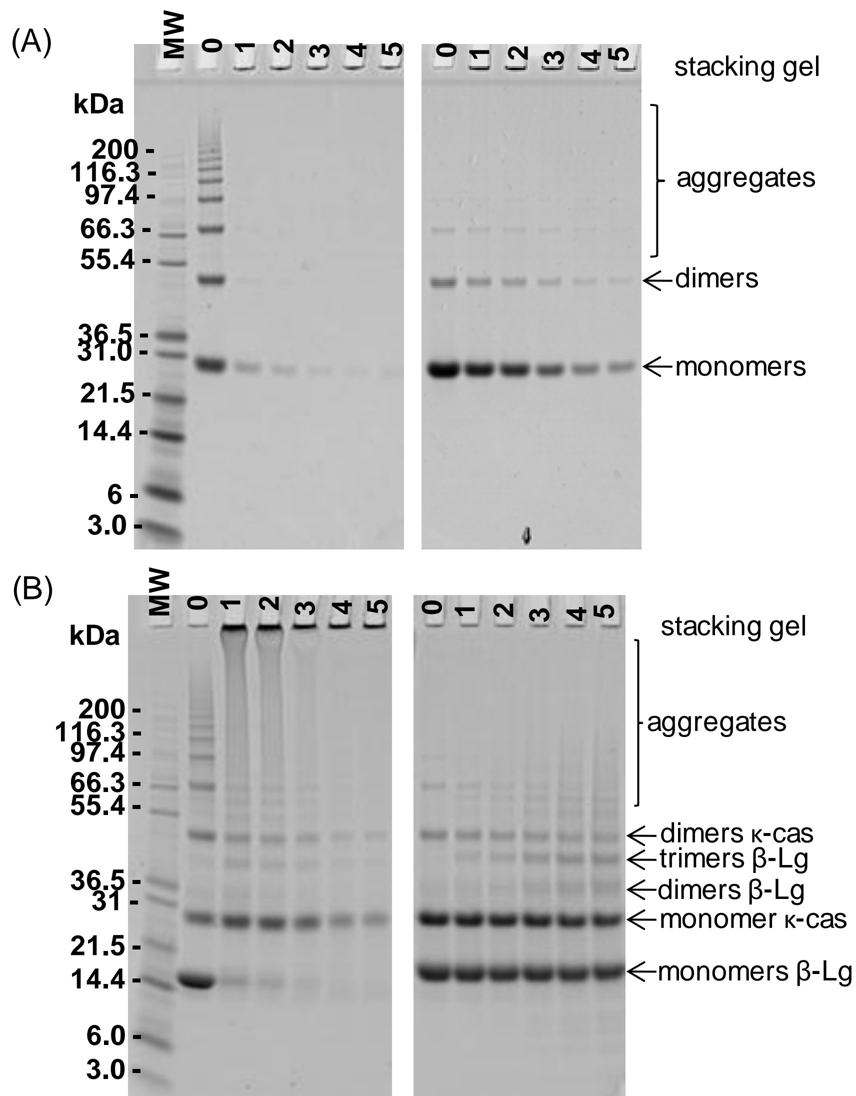

Figure 2: LDS-PAGE of κ-casein (A) and (B) κ-casein + β-lactoglobulin, heated 90 ºC/2 h, non reduced (left) and reduced (right) with DTT. (0) Native protein and (1) 10 min, (2) 20 min, (3) 45 min, (4) 90 min and (5) 2h of heating. MW = Molecular Weight Marker

Even if it is not relevant, to measure particle size by dynamic light scattering, for fibrils (non spherical particles), such analysis was performed to compare the three heat treated samples (Table 1). Analysis showed that heating increased the average particle size which was also observed in PAGE and TEM analysis. An increase of average size was obtained after only 10 min heating for all samples. After that and during the course of heating, a progressive increase of the average size was observed for heat individual proteins. Interestingly, this progressive increase of particle size was not observed for other protein systems in which a constant average size around 30,

62 and 52 nm was found to α_{S2}-CN, β-Lg+ α_{S2}-CN and β-Lg + κ-CN, respectively, whatever the heating time.

The model used to DLS analysis is based in diameter of sphere, and changes at the shape of particle can affects the diffusion speed. However, Kokhanovsky and Jones (2002) proposed the cross-polarization scattering for the characterization of non-spherical large particles.

Table 1: Average particle size (Malvern Zetasizer) of β-lactoglobulin (β-Lg) with κ-casein (κ-CN) and α_{S2}-casein (α_{S2}-CN) heated 90 ºC for 2 h, separately or mixed

time (min.)	Average particle size (nm)				
	α_{S2}-CN	β-Lg + α_{S2}-CN	β-Lg	κ-CN	β-Lg + κ-CN
0	17.8±0.7	-	6.5±0.1	18.8±0.4	-
10	28.5±0.9	59.0±0.2	53.2±1.3	121.8±0.4	50.6±0.2
20	28.3±0.7	61.4±0.2	50.0±0.6	130.4±0.1	51.0±0.3
45	29.0±1.1	62.8±0.3	60.0±0.8	177.8±1.9	52.8±0.2
90	30.3±1.7	62.8±0.3	68.2±0.3	191.8±3.9	52.1±0.1
120	32.6±0.5	63.4±0.3	70.0±0.4	190.5±3.4	52.8±0.1

TEM images completed these results and showed the morphology of the aggregates produced. We can observe that both, heat treatment and protein interaction change the morphology of aggregates. β-Lg heated 90 ºC for 2 h (Figure 3C) presented as a mixture of shorter twisted fibrils (less than 100 nm in length) and aggregates. α_{S2}-CN native and heated 90 ºC for 2 h (Figure 3B and 3D, respectively), presented as spherical aggregates, with a maximum of diameter ~ 40 nm, which was observed by Thorn et al. (2005) at 37 ºC. The images of heated mixed proteins (Figure 3E) showed the presence of ribbon-like fibers, with size between 50 and 150 nm.

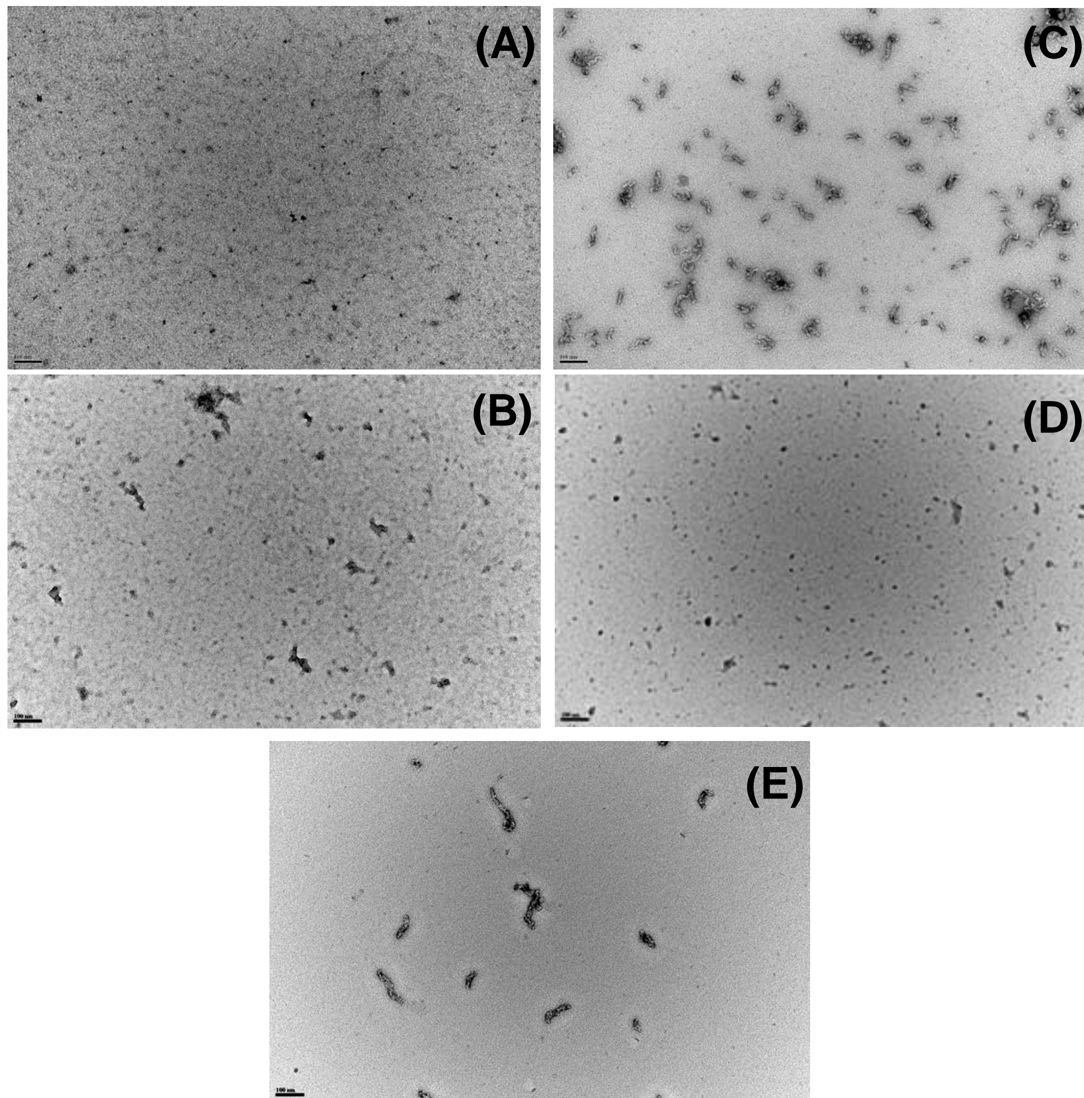

Figure 3: TEM images of native β-lactoglobulin (A) and native α_{S2}-casein (B) or heated β-lactoglobulin (C), α_{S2}-casein (D) and β-lactoglobulin + α_{S2}-casein (E) 90 ºC/2 h. Bars=100 nm

The formation of fibrillar structures was showed by ThT fluorescence analysis (Figure 4): the formation of fibers was observed in all heated samples with the same value of fluorescence at the end of heating.

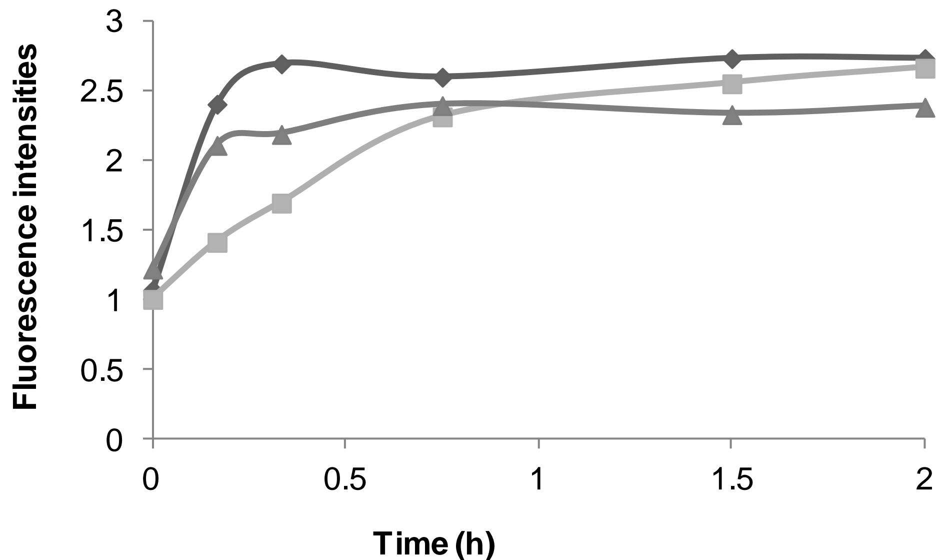

Figure 4: Thioflavin T (ThT) fluorescence of β-lactoglobulin (□), k-casein (◊) and β-lactoglobulin + k-casein (∆) solution heated at 90 °C and pH 7. The emission intensity was measured at 478 nm upon excitation at 446 nm.

TEM was used to further analyze the formed aggregates between the two proteins. Native κ-CN solution (Figure 5B) was composed by a variety of forms that were fibrillated after 2 h of heat treatment (Figure 5D). Fibril formation, rather than association into spherical aggregates, seems to be predominant for heated κ-CN as showed by Leonil et al. (2008). The heated mixture (Figure 5E) of κ-CN and β-Lg was composed by other structures of aggregates and κ-CN fibrils formation was reduced.

ThT fluorescence intensity of the heated β-Lg was identical to that of the heated κ-CN, 2h after heat treatment. However, the TEM pictures (Figure 5) are diametrically opposed in terms of the density of fibrils with a wealth of amyloid aggregates for κ-CN, which was not observed for β-Lg aggregates.

The discrepancy between the results of ThT and TEM can be explained by the technical limits of ThT put into question as to the specificity and affinity only to amyloid fibril of the thioflavin dye or to the inability of ThT to penetrate fibril bundles to reach binding sites (Cloe et al. 2011).

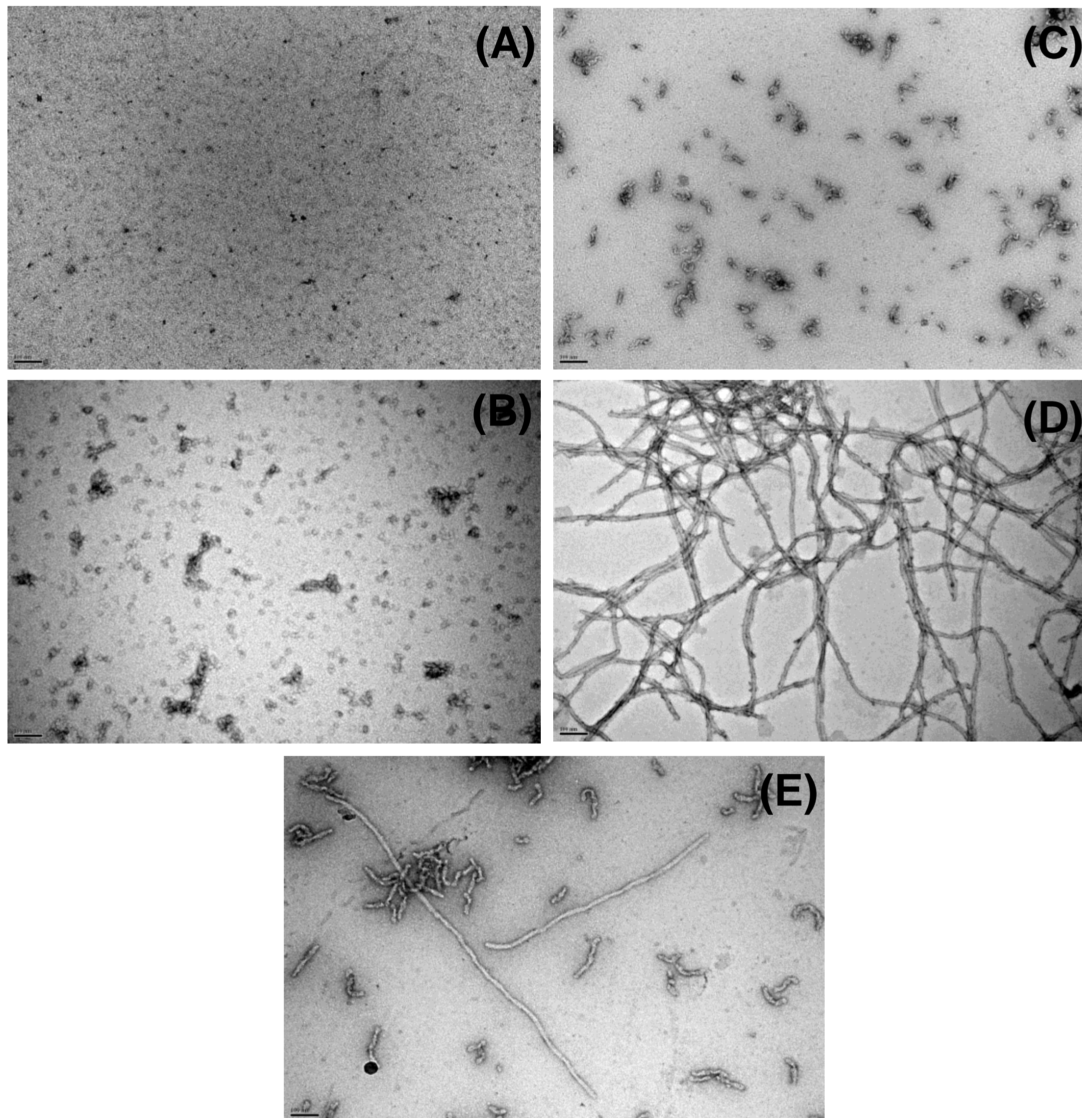

Figure 5: TEM images of native β-lactoglobulin (A), native κ-casein (B), heated (90 ºC/2 h, pH 7) β-lactoglobulin (C), heated κ-casein (D) and heated β-lactoglobulin+κ-casein (E). Bars=100 nm

In vitro Digestion of the Aggregates

After heat treatment of β-Lg, α_{S2}-CN and κ-CN aggregates were subjected to *in vitro* simulated gastro-intestinal digestion.

Figure 6 shows LDS-PAGE of gastric digestion of native and heated α_{S2}-CN. In the same gel we can observe the bands of the enzymes used during digestion. Heated α_{S2}-CN was more readily digested than native protein, despite in both cases proteins were completely digested in gastric phase. In these conditions, duodenal step was not needed. Complete digestion of α_{S2}-CN and its oligomers was achieved after 10 minutes incubation with pepsin.

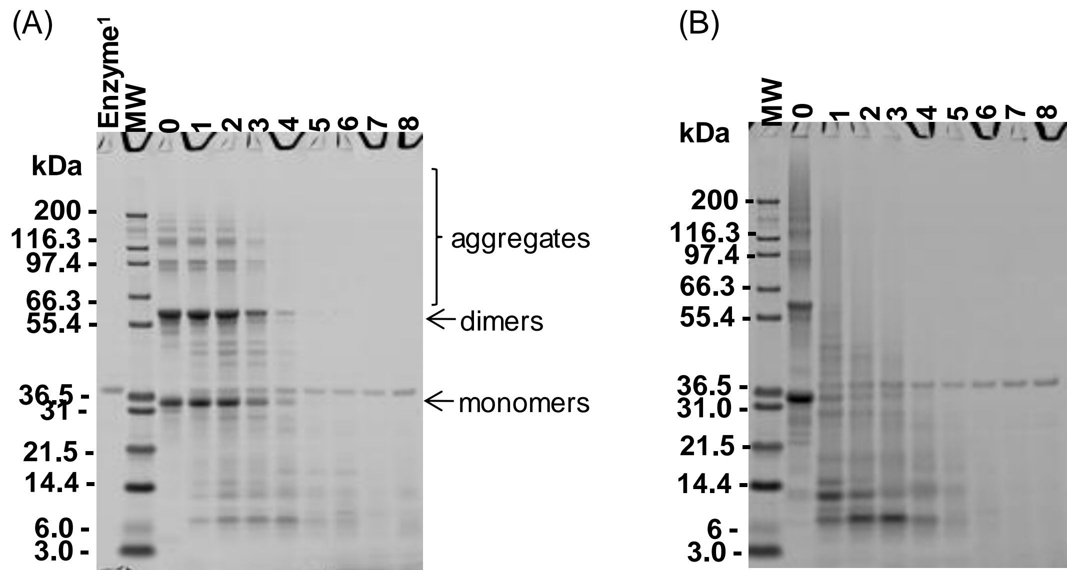

Figure 6: LDS-PAGE of native (A) and 90 ºC/2h heated (B) α_{S2}-casein in digested gastric. Profile without enzyme (0), or with enzyme for (1) 0 min, (2) 1 min, (3) 2min, (4) 5 min, (5) 10 min, (6) 20 min; (7) 40 min and (8) 60 min. MW = Molecular Weight Marker. Enzyme1: Pepsin

β-Lg *in vitro* digestion was analyzed by LDS-PAGE and gels were presented in Figure 7. As expected native β-Lg was found to more resistant to digestion than heated denature protein. Native form of the protein have resisted to gastric and duodenal digestion at the studied levels and only in duodenal phase it was possible to detecte the degradation of protein in some peptides bands in the electrophoresis gel. Large aggregates formed in heated β-Lg (90 ºC/2 h) were gradually broken down into small peptides (< 14 kDa) in gastric digestion. Digestion of aggregates with pepsin starts immediately after addition of the enzyme (line 0 min). In duodenal digestion larger peptides were broken into smaller peptides. In particular, peptides with molecular weight (Mw) around 6 kDa were completely hydrolyzed after 5 min. duodenal digestion. Similar hydrolytic pattern was obtained after gastric and duodenal digestion of mixed protein system.

It is interesting observe that the band of 2h heated β-Lg (Figure 1B) (line 5) was different that the band of the same sample at Figure 7 (line 0). It can be related with pH 2.5 of the simulated gastric fluid, which samples have subjected even before the addition of enzymes.

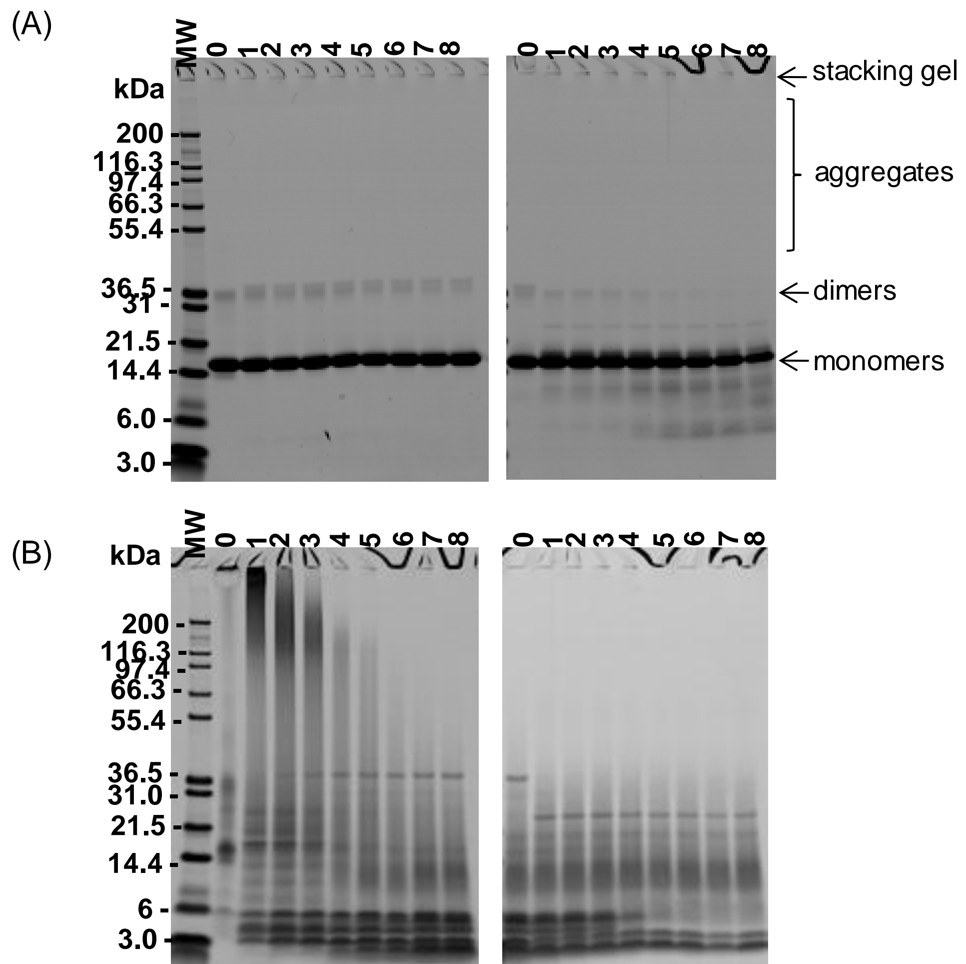

Figure 7: LDS-PAGE of native (A) and 90 ºC/2h heated (B) β-Lg digested gastric (left) and duodenal (right). Profile without enzyme (0), or with enzyme for (1) 0 min, (2) 1 min, (3) 2min, (4) 5 min, (5) 10 min, (6) 20 min; (7) 40 min and (8) 60 min. MW = Molecular Weight Marker

The results of *in vitro* digestion of native and heated κ-CN are shown in Figure 8. The bands of native sample disappeared immediately during gastric digestion. For heat treated sample, large aggregates are still observed at the top of the gel even after 1 h gastric digestion. During duodenal digestion only traces of large aggregates remained, however, few bands of peptides have appeared in the gel. It is possible that the produced peptides completely out of the gel or that the aggregates were diluted in the digestion solution (simulated gastric fluid or duodenal digestion components).

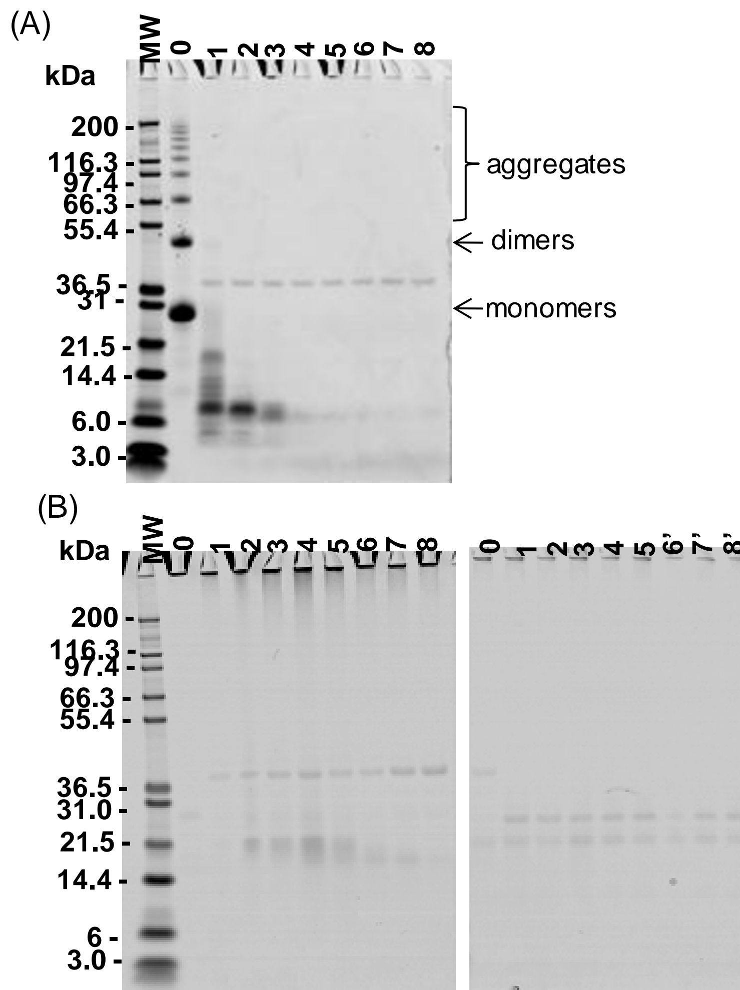

Figure 8: LDS-PAGE of native (A) and 90 ºC/2 h heated (B) κ-casein digested gastric (left) and duodenal (right). Profile without enzyme (0), or with enzyme for (1) 0min (2) 1 min, (3) 2min, (4) 5 min, (5) 10 min, (6') 15 min, (6 and 7') 20 min; (8') 30 min, (7) 40 min; (8) 60 min. MW = Molecular Weight Marker

The effect of heating on protein digestion was further quantified by monitoring the degree of hydrolysis (DH) as monitored throughout by TNBS method (measure of released free NH_2 groups). The results are reported in Figure 8 for β-Lg, κ-CN and the mixture of both. Native and heated κ-CN reached about 8% of DH at the end of digestion. Focusing on the gastric digestion step, we observed that native sample (Figure 9A) had a DH (7%) slightly higher than the heated (Figure 9B) sample (5%), in accordance with LDS-PAGE results. Hence, the formation of large aggregates seems to be decrease the sensibility of κ-CN to the digestion. Heated β-Lg was found to be

the more sensitive sample after gastric and duodenal digestion steps. Sample with mixed protein exhibited intermediary digestion behavior.

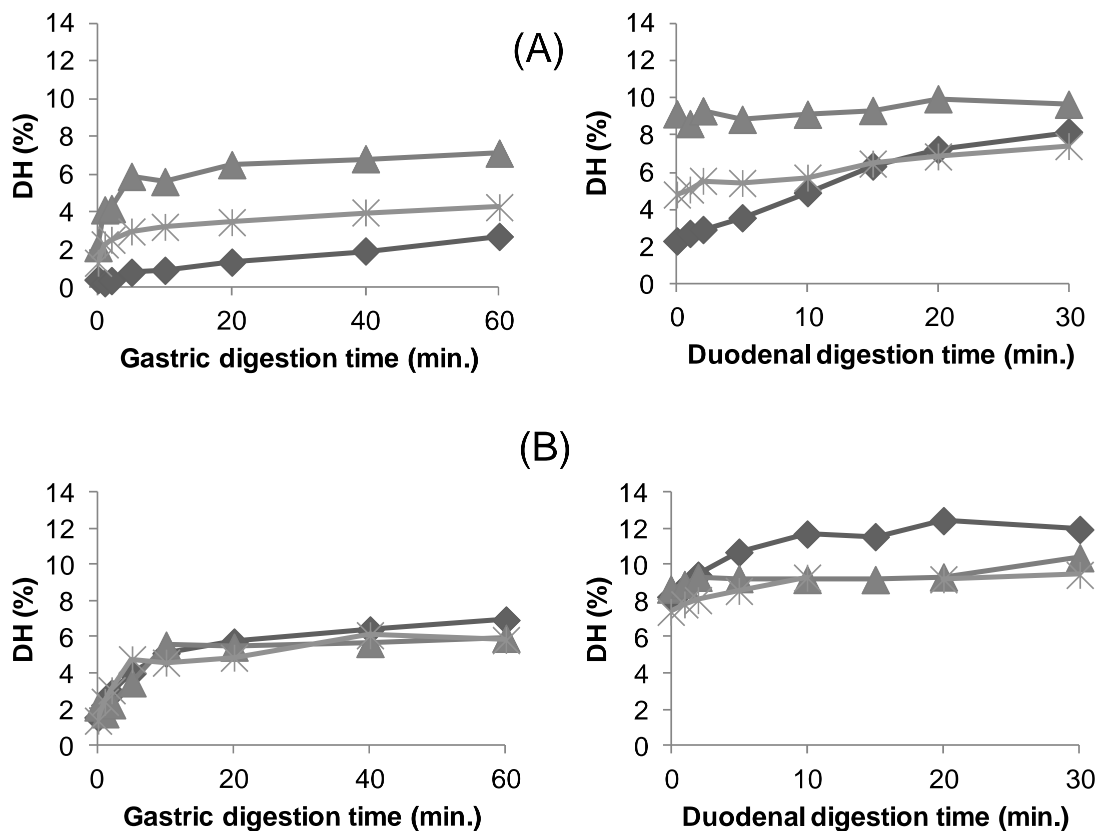

Figure 9: Degree of hydrolysis of gastric (left) and duodenal (right) digested native (A) and 2 h heated (B) proteins. (◊) β-lactoglobulin, (∆) κ-casein, (x) β-lactoglobulin+κ-casein. *maximum standard deviation: 0.082

The results of gastro-duodenal digestion of mixed proteins are shown in Figure 10. As α_{S2}-CN aggregates are digested faster, the overall profile (Figure 10A) resembles that of β-Lg heated alone. At the end of digestion steps, peptides with Mw around 6 kD completely disappeared while polypeptides with Mw of 10-12 kDa and others with Mw of 3 Kda were still present as in the case of β-Lg alone.

The bands of digested β-Lg + κ-CN aggregates also resembles that of β-Lg heated alone, since small κ-CN aggregates are rapidly digested and the biggest aggregates remained at the top of the gel, differently to the gel profile at Figure 10B.

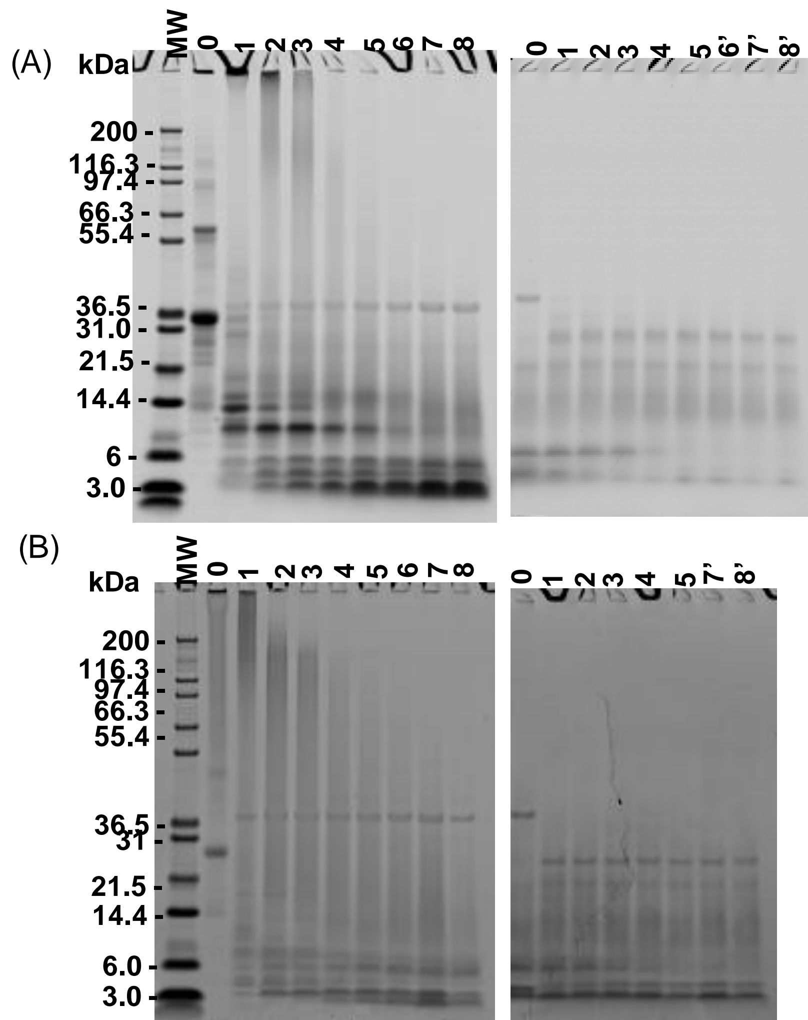

Figure 10: LDS-PAGE of β-lactoglobulin heated at 90 ºC/2h with α_{S2}-casein (A) and κ-casein (B) digested gastric (left) and duodenal (right). Profile without enzyme (0), or with enzyme for (1) 0min (2) 1 min, (3) 2min, (4) 5 min, (5) 10 min, (6 and 7') 20 min; (8') 30 min, (7) 40 min; (8) 60 min. MW = Molecular Weight Marker

DISCUSSION

It is known that β-Lg conformation change when heated, first unfolding and then the irreversible complexes may be formed (Verheul et al., 1998). Furthermore, intramolecular reaction in heated β-Lg must precede intermolecular interactions (Lowe et al., 2004). The whey proteins are highly structured, but the four caseins lack stable secondary structures. This limited folded structure of caseins play an important role during heat treatment since caseins are stable and hardly change during heat treatment.

We studied the heat induced aggregation of have studied the characteristics of the reaction between β-Lg, α_{S2}-CN and κ-CN in separate and mixed solutions.

The structural malleability of casein proteins facilitates their interaction with a destabilized protein (unfolded) which is in accordance with their chaperone activity (Morgan et al., 2005). In contrast to β-Lg and also k-CN, only oligomers were formed when α_{S2}-CN was heated at 90°C and pH 7, probably due to its limited folded structure (Kohno et al., 1994). This is rather unexpected since, as in the case of the two other protein, α_{S2}-CN also possess two cysteine residues that could strengthen the aggregation process.

In the case of κ-CN, when the protein was heated alone, the intensity of monomers and dimers bands in LDS-PAGE analysis were reduced during the holding time to form large aggregates. Up to 10 min of heating, κ-CN solution was mainly composed by large aggregates with a variety of structural forms. After 2 h of heat treatment, TEM images showed that the sample contained essentially fibrillar structures reaching up to 900 nm or more in length and 10-12 nm of width.

When the two proteins were mixed in a stoichiometric ratio (β-Lg + α_{S2}-CN or β-Lg + κ-CN) after heating we found by LDS-Page, a speed up in the heat-induced aggregation in mixture which was followed by faster disappearance of β-Lg monomer bands. This must be related to the cysteine exposure when β-Lg lost secondary conformation. Caseins presents limited secondary structure, and they cystein residue can readily react with monomers after β-Lg denaturation. This feature was presented by Morand et al. (2011) to the β-Lg + κ-CN model.

Furthermore, part of κ-CN monomers and dimers are still present even after 2 h of heating. These results arise de question about the nature of formed dimers in mixed protein solution. The formation of heterodimers between β-Lg and κ-CN has been previously suggested (Cho et al., 2003). Other experiments, using proteomic tools, are needed to confirm the cross interaction and a co-aggregation between β-Lg and κ-CN in model system as such interaction occurs in heated milk.

The formed aggregates were mainly linked by covalent bonds such as disulfide bonds. Cho et al. (2003) proposed that when β-Lg and κ-CN were

heated together, β-Lg formed thiol-exposed monomers, which can reacted with each other or with the native k-CN. These authors, founded some disulphide-bonded 1 : 1 b-lg : k-CN complexes, some monomer k-CN and a range of large aggregates held together by either or both disulphide bonds and hydrophobic association. However, other nature of covalent bonds are showed in our work, and it can be due to the hard and intense heat treatment (Gulzar et al., 2011). The last bonds were not reduced even after a long time in contact with DTT. These covalent bonds could be attributed to the formation of covalent isopeptide bounds between protein molecules as already suggested by Mudgal et al. (2011) during purification or storage steps or even a succinimide formation, as proposed by Desfougères et al. (2011) for dry-heated lysozyme.

After reduced with DTT, bands of monomers in mixed samples were observed, like when the proteins were heated alone. Furthermore, bands like β-Lg trimers (~ 51 kDa), was also observed in the reduced gel, both in β-Lg and β-Lg + α_{S2}-CN gel. These trimmers could be involved in the aggregates and were not reduced in the presence of DTT. This feature showed that β-Lg/α_{S2}-CN aggregates could be formed mainly by disulfide bonds from monomers of these proteins, and also by trimmers of β-Lg, which was favored in the mixed system. Nevertheless, the occurrence of a covalent heterodimer β-Lg/α_{S2}-CN that migrates at the same position as β-Lg trimer band cannot be ruled-out.

The presence of β-Lg also affects the length and number of κ-CN fibrils as assessed by TEM images (Figure 4E). Guyomarc'h et al., (2009) suggested that the addition of κ-CN can lead to a reduction in the charge density of β-Lg and consecutively to a reduction of aggregates size. In this case, κ-CN would form aggregates with β-Lg which are not available to lead into fibrillar structures. In the mixed systems (κ-CN with β-Lg) the reaction seemed faster than when protein was heated alone, due to the faster decrease of the bands at the LDS-Page. This must be related to the cysteine exposure when β-Lg lost tertiary conformation. Furthermore, high temperatures have favored the formation of covalent bonds, others than disulphide bonds as already reported under other heating conditions (Gulzar et al., 2011).

Analysis of hydrodynamic radius showed that after 90 min heating, β-Lg aggregates were greater than the β-Lg/α_{S2}-CN aggregates and by TEM images we found that fiber-like structuress were produced under heating. However, the

form of fibers was different i.e. like twisted fibers for β-Lg aggregates and like ribbon for β-Lg/α_{S2}-CN aggregates. Thorn et al. (2008) showed that spherical particles typical of α_{S2}-CN rapidly converted to twisted, ribbon-like fibrils (neutral pH, 37 ^{o}C), under stirring.

After 10 min, the biggest particles were formed with κ-CN heated alone. After that and during the course of heating, a progressive increase of the average size was observed for heat individual proteins. Interestingly, this progressive increase of particle size was not observed for mixed protein system in which a constant average size around 52 nm was found whatever the heating time.

Briefly, heat treatment promotes changes in the structure and aggregation of the studied proteins, which was observed by some techniques used in this work, i.e., LDS-PAGE, SEC and TEM. However, the methods are insufficient to assure if hetero or homo-aggregates were produced, at mixed systems.

In vitro digestion of the aggregates

In the study of α_{S2}-CN and β-Lg, we observed that heated proteins are more readily digested than native proteins. Furthermore, the LDS-PAGE profile of the mixture was similar to the β-Lg. However, it was expected different profile (more enzyme sensitive) due to the interaction of these proteins that have prevented β-Lg aggregation. We have observed that when β-Lg was less associated (10 min. of heating; for example), the resistance to digestion was smaller (data not show). This feature, confirm one more time, that cross interactions can be preferable than single interactions, when α_{S2}-CN and β-Lg were heated together. And these interactions can change the action of the digestion enzymes, due the changes at the specific site of enzymes.

As expected, *in vitro* experiments showed that κ-CN (unstructured protein) was more sensitive to digestive enzyme than β-Lg, more compact and globular protein. Peptides with intermediary molecular weight (3 kDa>Mw>14 kDa) were detected for digested β-Lg while digested κ-CN was totally converted to very small peptides, with Mw below 1 kDa, undetected in the electrophoresis gel.

Also, the heat-induced aggregates of κ-CN or β-Lg + κ-CN are more sensitive to gastric digestion that native samples, they were easily digested and

also presented a higher DH. Remarkably, in the case of κ-CN heated alone, some formed aggregates resisted to digestion. This was less the case for β-Lg aggregated structures. About the lower number of peptides identified at κ-CN digested gels, Dupont et al. (2010a) explained that this is a result of a higher degree of proteolysis, leading to short undetectable peptides.

CONCLUSION

Milk serum and casein transformation when subjected to heat treatment is different if they are alone or mixed in solution. Since these proteins have high value and is largely used as ingredients in food industry and other, the study of the changes becomes important.

In this work, we showed the changes in β-Lg in presence of α_{S2}-CN and κ-CN. The whey protein, have formed smaller aggregates when in presence of these caseins and κ-CN forms nice fibrils after heating, the length of which was reduced in the presence of β-Lg. These aggregates were formed by disulphide bonds mainly; however other covalent bonds, which were not reduced in presence of DTT, were produced in α_{S2}-CN+β-Lg systems. These covalent bonds were observed when κ-CN was heated alone, however in presence of β-Lg, these bonds were prevented.

Aggregates in mixed system are readily digested by gastric enzymes. Differently, high aggregates of κ-CN heated alone were resistant to gastric digestion in the studied conditions.

Some evidences exist on the co-aggregation of these proteins, nevertheless further studies are needed to confirm such process.

These fundamentals studies highlighted some possible interactions between β-Lg and these two caseins, during heat treatment, and give more detail to the possible transformations in dairy process, such as yogurt and other milk-based drinks which are subject to similar heat treatment. Future studies will be conducted to determine the biological impact, e.g. allergenicity of co-aggregated structures and of the product of their digestion.

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5. GENERAL DISCUSSION

The challenge of ongoing studies is to better understand how protein-protein interactions are affected by reactions that can be encountered during food processing. Now heat treatment is amongst the major processes used to ensure safety as well as to create functionalities. The aim of this work was to demonstrate the effect of different partners (proteins, sugar) on the heat-aggregation of milk proteins. First, we have demonstrated the influence of glucose on: a major whey protein, β-lactoglobulin (β-Lg) and a major casein in bovine milk, β-casein (β-CN), a well-structured and a random coil protein, respectively over a strong heat treatments (90 °C). Moreover, we showed how β-Lg interaction was affected by κ-casein (κ-CN) and α_{S2}-casein (α_{S2}-CN) and conversely, how β-Lg affect κ-CN or α_{S2}-CN interaction. Finally, we studied the sensibility of aggregates formed under conditions to an *in vitro* simulated digestion.

In our present work, we confirmed that β-Lg is able to fibrillate at pH 7 following heating. The twisted fibrils are long (up to 900 nm) with a width of about 20 nm. The shape of β-Lg fibrils is similar to that already reported at the same pH by Jung et al. (2008). The morphology that we observed may explain why some material was accumulated within the wells of the stacking gel during PAGE analysis. Interestingly, this means that the β-Lg fibrils were not completely dissociated by LDS detergent. These results are in agreement with those reported by Bolder et al. (2007). The addition of an excess of reducing sugar, namely glucose, before heating induced the formation of AGE. The progressive association of monomers into oligomers and then into small aggregates is clearly observed in the presence of glucose, as opposed to the rapid formation of very large aggregates in the control sample influenced the β-Lg fibrillation process. A similar effect of β-Lg fibrillation by κ-carrageenan, an anionic polysaccharide, at pH 2 was recently reported (Jones et al., 2010).

In the study of a rondom coil structured protein, β-CN, our results showed that β-CN hardly change during heat treatment due a loosely structure. We can also observe, by TEM images, the differences between glycated and no-glycated samples. β-CN heated 90 ^{o}C for 24 h is seen as spherical species with a diameter about 10-20 nm.

When β-CN was heated in presence of glucose, AGE intensities relative to the protein concentration proved the formation of products of the Maillard

reaction. In LDS-PAGE analysis, band of aggregates appeared faster when β-CN was heated without glucose. In glycated β-CN covalent bonds were produced, as described by Pellegrino et al. (1999). They showed large aggregates progressively accumulated at stacking gel in glycated sample. Furthermore, in our work, we showed the formation of covalent aggregates also in no-glycated β-CN samples during heating time. This aggregates entered on the electrophoresis gel, however they have not dissociated by LDS-PAGE. Another important group of chemical modifications that occur in protein during heating, it results from β-elimination and condensation leading to protein crosslinks (Gerrard, 2002). However Pellegrino et al, (1999) fail to determine the nature for intermolecular protein crosslinking and found a minor role for the formation of lysinoalanine, lysylpyrraline and a possible role for pentosidine (see Figure 1, page 12). The mechanism causing protein aggregation under these conditions is currently undetermined.

The morphology of the β-CN aggregates, which have presented like a round species is different to the β-Lg aggregates, similar to the fibers. Besides, 24 h-glycated β-CN maintained the spherical shape but presented a greater diameter (20 – 50 nm).

The study on the interaction between B-Lg and α_{S2}-CN is at our knowledge quite new as far as we know. Reaction between β-Lg and α_{S2}-CN presented some particularities that can give more information about their interactions. By LDS-PAGE it was possible to note a speed up in the heat-induced aggregation in β-Lg + α_{S2}-CN system which was following by faster disappearance of β-Lg monomers and α_{S2}-CN dimmer band. Some elements will tend to indicate that β-Lg/α_{S2}-CN aggregates was formed, mainly linked by covalent bonds, such as disulfide bonds or other due the hard and intense heat treatment. The last one did not reduce even after a long time and contact with DTT. These covalent bonds could be attributed to the formation of covalent isopeptide bounds between protein molecules as already suggested by Mudgal et al. (2011) during purification or storage steps or even a succinimide formation, as proposed by Desfougères et al. (2011) in their work with lysozyme. TEM images showed that fibers were produced under heating. However, the form of fibers was different, like twisted fibers to β-Lg aggregates and like ribbon to postulated β-Lg/α_{S2}-CN aggregates. Thorn et al. (2008)

showed that spherical particles typical of α_{S2}-CN rapidly converted to twisted, ribbon-like fibrils (neutral pH, 37 ºC). These fibrils possessed a beta-sheet core structure and the ability to bind to thioflavin T. Therefore, interaction among different proteins was predominating. α_{S2}-CN produced complexes with denatured β-Lg, which prevented further aggregation of β-Lg.

In the study of κ-CN and β-Lg taken as a reference model, our results are in accordance with the literature. Native κ-CN have presented as a mixture of monomers, dimmers and aggregates species. Up to 10 min. of heating, κ-CN solution was mainly composed by large aggregates, composed by a variety of forms that was fibrillated after 2 h of heat treatment. Thorn (2005) demonstrated that κ-CN heated 37 °C for 180 h shows the presence of fibrils of up to 200 nm. Furthermore, Leonil et al. (2008) pointed out the key importance of the N-terminal domain of κ-casein in the fibrillation process.

In mixed systems, we have showed a reduction in the length and number of κ-CN fibrils produced. This was in accordance with Cho et al. (2003), which proposed that when mixtures of β-Lg and κ-CN were heated, the free thiol of β-Lg was exposed and this initiated a series of thiol–disulfide exchange reactions of β-Lg with other denatured β-Lg molecules or with κ-CN. Furthermore, Guyomarc'h et al. (2009) suggested that the addition of κ-CN can lead a reduction in charge density of the β-Lg and consecutively a reduction of aggregates size. Now the formation of mixed fibrils is in debate and highly questionable by the mechanism of fibril formation (Maji et al., 2009; Hall and Huang et al., 2012). Our study shows an effect on the length of κ-CN fibrils but belongs no evidence on the formation of such mixed fibrils.

These four studied protein interactions (β-Lg and β-CN with glucose; β-Lg with κ-CN and α_{S2}-CN) were submitted to *in vitro* adult digestion model as described by Dupont et al. (2010b).

Formation of milk protein aggregates and products of advanced Maillard reaction could be responsible for changes to proteolysis. Milk processing led to differences in peptide patterns with an increase in the number of peptides found in digested samples. This modification can be linked to the allergenic potential of milk products (Dupont et al., 2010a). It is know the high allergenicity potential of cows' milk, mainly the caseins α_{S1}-, α_{S2}-, k- and β-CN and β-Lg are

considered to be a major cause of allergic response to cows' milk (Wal, 2001; Mantyjarvi et al., 2000).

Native β-Lg resists to 90 min-gastric/duodenal digestions being almost unaltered. In the other hand, native β-CN was readily digested already in gastric digestion and these results are in accordance with other works (Mandalari et al., 2009; Dupont et al., 2010a; Barbé et al., 2013). When proteins are heated, digestion resistances are changed. Generally, β-Lg lost the native resistance and β-CN becomes more resistant. Heated β-Lg was described as twisted fibrils, which in some cases have been described as a form of aggregation that are stable and resistant to degradation by proteases (Ecroyd et al., 2008). However, in this work, we can observe that β-Lg twisted fibrils were broken down into small peptides.

We have also presented that glycated samples were more resistant to digestive enzymes than no-glycated one at *in vitro* digestion model. Corzo-Martinez et al. (2010) have showed this feature to glycated β-Lg.

In protein/protein studies, we have observed that mixed aggregates are readily digested by gastric enzymes, as well α_{S2}-CN aggregates, contrary to κ-casein fibrils aggregates that were resistant to the *in vitro* digestion.

6. GENERAL CONCLUSION AND PROSPECTS

The present results give new insight into the complex aggregation/fibrillation pathway of some milk proteins at neutral pH and on how the process is affected by an environment that can be encountered in foods. Additional studies could be interesting with other techniques to identify more characteristics behind this aggregation, such as, X-ray or neutron scattering, differential scanning calorimetry, immune assay or fluorescence spectroscopy with use of specific dye to bind at different proteins. Furthermore, nutritional and functional consequences of the produced aggregates could be evaluated.

Our results about aggregates compounds could be explored for future understanding of controlled protein self-assembly processes, which is related to pharmaceutical interests, like controlled delivery of substances, such as bioactive compounds. This could also meet rising demand for diversification of final products properties e.g. sensory and nutritional properties. Furthermore, this study could be explored for the possible transformations in dairy process, such as condensed milk, doce de leite, infant formula, yogurt, milk-based drinks which are subject to similar conditions. The use of the aggregates may be interesting to diversify textural properties e.g. viscosity and shear thinning of formulated food products.

In vitro digestion results could contribute to the allergenicity studies of proteins and to the formulation of new products that optimizes nutrition of the consumer.

Moreover, these studies clarify the necessity to improve the techniques to characterize aggregates produced mainly in mixed systems. Given the complexity of the aggregates formed the wide diversity in their size, properties and morphologies, the current methods failed to better characterize the resulting assembled supramolecular structures. This is especially true in mixed protein systems as it is the case in the present work. Methodological improvements are needed to overcome the limit of generally used methods as recently discussed by Hall and Huang (2012).

Beyond these features, this thesis proposes a reflection on the excessive use of heat treatment in milk products, with a focus in one of more important components of milk, the proteins

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