**CHARACTERIZATION AND IMPROVEMENT OF RHEOLOGICAL PROPERTIES OF SODIUM
CASEINATE GLYCATED WITH GALACTOSE, LACTOSE AND DEXTRAN**

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Abstract

The aim of this work was to investigate the effect of non-enzymatic glycosylation with galactose, lactose, and 10 kDa dextran on the rheological properties of sodium caseinate. To promote the formation of covalent complexes, the reaction was done in solid state ($a_w=0.67$), pH 7.0 (0.1 M sodium phosphate buffer), and temperature set at 50 and 60°C. The progress of Maillard reaction was indirectly traced by measuring the formation of the Amadori compound, through furosine (2-furoylmethyl-lysine) analysis, and brown polymers, and the resulting glycoconjugates were characterized by LC/ESI-MS and SEC. Results showed a higher reactivity of galactose than lactose and dextran to form the glycoconjugates, due to its smaller molecular weight. Glycation with galactose and lactose increased the viscosity of caseinate and also altered its flow characteristics from Newtonian to shear-thinning. Oscillatory testing showed a higher elastic modulus (G') in glycoconjugates when compared to non-glycated caseinate, especially with galactose, where a gel like behavior was observed after long incubation times. Glycation with dextran did not produce substantial improvements in the rheological properties of caseinate, probably due to the limited extent of the reaction. Our results show that by controlling the rate and extent of the Maillard reaction is a technologically feasible operation to improve the viscosity and gelling properties of sodium caseinate-based ingredients.

Keywords: sodium caseinate; carbohydrates of different molecular weight; non-enzymatic glycosylation; neoglycoproteins; rheological properties.

1. Introduction

Sodium caseinate (SC), a more soluble form of casein, is extensively used in food industry as a functional ingredient in a wide variety of food products due to its simple production, excellent nutritional value, and versatile functional properties, including heat stability, water-holding capacity (WHC), fat binding, rheological properties (increase of viscosity and gel formation) and foaming, emulsifying, and stabilizing capacities. Concretely, WHC and gelling properties are used to improve rheological properties, texture, stability, and appearance of many food products such as processed meats, surimi, cheese, yogurt and confectionary products. However, SC is required in relatively high concentrations to produce viscous dispersions (Ennis & Mulvihill, 2000), resulting in an expensive component in food formulations. Hence, improvements in its rheological properties could produce an equivalent functionality with a smaller amount of added protein, helping to reduce production costs. Initial attempts to improve the viscosity of SC by reaction with carbohydrates were done by Colas, Gobin and Lorient (1988) and Courthaudon, Colas and Lorient (1989). Nevertheless, the formation of these covalent glycoconjugates was achieved following an alkylation reaction with a solution of cyanoborohydride, a highly toxic agent, making the process unsuitable for food application.

Consumers demand for food ingredients with improved and desirable functional attributes is increasing and the food industry is moving towards the search of procedures to obtain new multifunctional ingredients. There is substantial evidence that controlled heating of proteins with reducing sugars of different molecular weight to form glycoconjugates via the Maillard reaction (so called neoglycoproteins), is one of the most efficient and safest methods to generate new functional proteins with great potential as novel ingredients (Oliver, Melton & Stanley, 2006a). Extensive glycation has been shown to decrease the solubility of proteins due to polymerization and further cross-linking, produced at the most advanced stages of the reaction (Aoki, Hiidome,

Sugimoto, Ibrahim & Kato, 2001; Katayama, Shima & Saeki, 2002; Sato, Sawabe, Kishimura, Hayashi & Saeki, 2000; Tanabe & Saeki, 2001), whereas the viscosity and gelling properties can be improved by carefully controlling the extent of the Maillard reaction.

Under controlled conditions, globular proteins of different nature (e.g., whey and egg white proteins, soy protein isolate) have been conjugated via the Maillard reaction with xylose, ribose, glucose, fructose, lactose or galactomannan, to improve their gelling properties (Hill, Mitchell & Armstrong, 1992; Armstrong, Hill, Schrooyen & Mitchell, 1994; Cabodevila, Hill, Armstrong, De Sousa & Mitchell, 1994; Easa, Hill, Mitchell & Taylor, 1996; Handa & Kuroda, 1999; Rich & Foegeding, 2000; Lauber, Klostermeyer & Henle, 2001; Matsudomi, Nakano, Soma & Ochi, 2002; Sun, Hayakawa & Izumori, 2004). However, despite the fact that caseins lack a complex secondary and tertiary structure (Farrel, 1988) and could readily react with reducing sugars via the Maillard reaction, to the best of our knowledge, hardly any research has been conducted on this regard. Only recently, Oliver et al. (2006b) glycated SC with ribose, glucose, fructose, lactose and inulin via the Maillard reaction with the aim of describing the effect of reaction conditions, including relative humidity, and type and concentration of sugar, on viscosity of SC. These authors reported interesting results, as SC glycoconjugates showed to possess an increased viscosity compared to unmodified SC, as well as a non-Newtonian behavior. Nevertheless, no further studies on the effect of glycation on the viscoelasticity of SC were carried out. In this sense, the knowledge on viscoelastic properties is needed to completely characterize the rheological behavior of SC glycoconjugates, which will largely determine the quality of foods to which they are added (Herh, Colo, Roye & Hedman, 2000; Ramaswamy, Basak, Abbatemarco & Sablani, 1995). Moreover, to the best of our knowledge, there is no available information on the relationship between rheological properties of SC and structural modifications in native protein induced by glycation with sugars of different molecular weight, including polysaccharides.

The aim of this work was to investigate the influence of the sugar molecular weight on the structural changes of SC following glycation via the Maillard reaction with galactose (Gal), lactose (Lac) and 10 kDa dextran (DX), as well as to evaluate the effect of these changes on the flow and viscoelastic properties of SC.

2. Materials and methods

2.1. Preparation of sodium caseinate – galactose /lactose / dextran conjugates

Dextran with average molecular weight of 10 kDa, produced by *Leuconostoc mesenteroides* strain no. 151-877 was dialyzed (Spectra/Por[®] 3 Biotech Dialysis Membrane Tubing, 3.5 kDa molecular weight cut-off, Spectrum Europe, Breda, The Netherlands) versus deionised water to remove low molecular weight oligosaccharides. Later, protein-reducing sugar combinations were prepared at the following weight ratios: SC:Gal/Lac at 1:0.2 (600 mg SC:120 mg Gal/Lac per portion) and SC:DX at 1:2 (600 mg SC:1.2 g DX per portion). For this, carbohydrates (Gal, Lac or DX) (Sigma-Aldrich, St. Louis, MO) and SC (Rovita FN 5, Proveedora Hispano Holandesa, S.A., Barcelona, Spain) were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 (Merck, Darmstadt, Germany) and frozen at -20°C for their subsequent lyophilization. The protein-carbohydrate powders were kept at 50 and 60°C for 96 and 24 hours, respectively, under vacuum in a desiccator equilibrated at 67% of relative humidity (RH) (Oliver et al., 2006b), achieved with a saturated solution of CuCl (Sigma-Aldrich, St. Louis, MO). At the equilibrium, the a_w in the powders was then 0.67. In addition, control experiments were performed with SC stored at 50 and 60°C without reducing sugars during the same periods (control heated SC). Incubations were performed in duplicate, and all analytical determinations were performed at least in duplicate. After incubation all samples were stored at -20 °C until used in the following analysis.

2.2. Assessment of the Maillard reaction evolution

The initial steps of Maillard reaction (formation of the Amadori compounds) were indirectly assessed through the determination by ion-pair RP-HPLC of the corresponding 2-furoylmethyl amino acid (2-FM-AA) after the acid hydrolysis of the dry-heated products. Briefly, 8.7 mL of 8 N HCl was added to 54.6 mg of SC in hydrolysis tubes and heated at 110°C for 23 h under inert conditions (helium), followed by the filtration through Whatman no. 40 filter paper. The filtered hydrolysate (500 µL) was applied to a previously activated Sep-Pak C18 cartridge (Millipore Corp., Billerica, MA, USA). Compounds retained in the cartridge were eluted with 3 mL of 3 N HCl, and 50 µL were used for injection (Moreno, López-Fandiño & Olano, 2002). Analysis was carried out via an ion-pair RP-HPLC method using a C₈ (Alltech furosine-dedicated; Alltech, Nicolasville, KY) column (250 x 4.6 mm i.d.) and a variable wavelength detector at 280 nm (LDC Analytical, SM 4000, Salem, NH). Operating conditions were as follows: column temperature, 35 °C; flow rate, 1.2 mL/min; solvent A, 0.4% HPLC grade acetic acid in double-distilled water; solvent B, 0.3% KCl in solvent A (Resmini, Pellegrino & Batelli, 1990). Calibration was performed by using known concentrations (0.52 to 5.2 mg/L) of a commercial pure standard of furosine (Neosystem Laboratories, Strasbourg, France). Data were expressed as mg per 100 mg of protein.

Browning of SC:Gal/Lac/DX conjugates (30 mg/mL in double-distilled water) was measured at room temperature by absorbance at 420 nm in a Beckman DU 70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA), as an index of the brown polymers formed in more advanced stages of non-enzymatic browning (Ting & Rouseff, 1986). Some insoluble conjugates were solubilized with sodium dodecyl sulfate (SDS) at 2.5% + heating at 70 °C for 10 min, before absorbance measure.

2.3. Structural characterization of glycoconjugates

Before LC/ESI-MS separations, dry-heated samples were dissolved in 1 mL of 0.1 M Tris/HCl pH 7.0 buffer (Sigma-Aldrich, St. Louis, MO) containing 8 M urea (Riedel-de Haën, Seelze, Germany) and 20 mM dithiothreitol (DTT, Sigma-Aldrich, St. Louis, MO) to give a final protein concentration of 10 mg/mL. After standing at 37°C for 1 hour, samples were diluted (1/3) with solvent A and filtrated with PVDF membranes (0.45 µm, Symta, Madrid, Spain). LC/ESI-MS experiments were carried out on a Finnigan Surveyor pump with quaternary gradient system coupled in-line to a Finnigan Surveyor PDA photodiode array detector and to a Finnigan LCQ Deca ion trap mass spectrometer using an ESI interface. Sample injections (7 µL) were carried out by a Finnigan Surveyor autosampler. All instruments were from Thermo Fisher Scientific (San José, CA, USA). RP-HPLC separations were carried out with a BioBasic-4 (100 mm x 2.1 mm, 5 µm) column (Thermo Fisher Scientific) at 25 °C using an adaptation of the method by Neveu, Mollé, Moreno, Martin and Léonil (2002). Separation was achieved at a flow rate of 200 µL/min and using 0.25% (v/v) of formic acid (analytical grade, Merck, Darmstadt, Germany) in double-distilled water (Milli-Q water, Millipore, Bedford, USA) as solvent A and 0.25% (v/v) of formic acid in acetonitrile (LC–MS Chromasolv® grade, Riedel-de Haën, Seelze, Germany) as solvent B. The elution program was applied as follows: at the start 20% B; after 2 min the percentage of B was linearly increased to 40% in 3 min; 40-50% B linear from 5 to 12 min; 50-80% B linear from 12 to 15min; 80% B isocratic from 15 to 20 min; ramped to original composition in 1 min; and then equilibrated for 15 min. The PDA detector operated in the wavelength range 190–600 nm in order to gather UV-spectral data. The detection wavelength was set at 280 nm. The mass spectrometer spray voltage was set at 4.5 kV, heated capillary temperature at 220 °C, nitrogen (99.5% purity) was used as sheath (0.6 L min⁻¹) and auxiliary (6 L min⁻¹) gas, and helium (99.9990% purity) as the collision gas. Mass spectra were recorded in the negative ion mode. The LC–MS system,

data acquisition and processing were managed by Xcalibur software (1.2 version, Thermo Fisher Scientific). Spectra of the protein peaks detected were deconvoluted using the BIOMASS deconvolution tool from BioWorks 3.1 software (Thermo Fisher Scientific).

With the aim to study the aggregation state, size exclusion chromatography (SEC) of glycated SC was carried out under nondenaturing conditions (0.05 M sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl) using a 24 mL Superdex 75 column HR 10/30 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) on an FPLC system. 100 μ L of a 1 mg/mL sample was applied to the column at room temperature. Elution was achieved in isocratic mode at 0.8 mL/min for 30 min and detection of eluting proteins was performed at 214 nm. The standard proteins used for calibration were human serum albumin (67 kDa), ovalbumin (43 kDa), α -chymotrypsinogen (25 kDa) and ribonuclease A (13.7 kDa) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The void volume was determined with Blue Dextran 2000. Additionally, native SC (10 mg) was subjected to denaturing conditions with 1 mL of 0.1 M Tris/HCl pH 7.0 buffer (Sigma-Aldrich, St. Louis, MO) containing 8 M urea (Riedel-de Haën, Seelze, Germany) and 20 mM dithiothreitol (DTT, Sigma-Aldrich, St. Louis, MO) and heated at 37 °C for 1 hour prior to SEC analysis performed under the conditions described above. Considering that: i) SEC separates on the basis of the hydrodynamic volume and not of the molecular weight, and ii) all the standard proteins used for calibration had a globular structure, the term M_r (relative molecular mass) instead of absolute molecular mass will be used in the Results and Discussion section when SEC analyses are described.

2.4. Rheological measurements

Control heated and glycated SC dispersions were prepared in distilled water at 30 mg/mL protein concentration for the determination of flow properties and at 150 mg/mL for viscoelastic

properties evaluation. Samples were left at room temperature ($20^{\circ}\text{C} \pm 2^{\circ}\text{C}$, ~ 16 h), with intermittent stirring, to allow complete hydration. Controls formed by SC and carbohydrate separately heated and mixed at the moment of analysis (control heated SC+carbohydrate), were also tested to study the effect of free carbohydrate on rheological properties of protein. The rheological measurements were performed using a controlled stress rheometer (model AR2000, TA Instruments, New Castle, DE) equipped with Peltier plate. The flow curves were obtained by monitoring shear stress (τ) as a response to 0 to 120 s^{-1} shear strain rate ($\dot{\gamma}$) ramp, with data recorded every 0.3 s during 1 min. All tests were performed at 25°C using the plate-plate geometry (plate, 40 mm diameter; 0.3 mm gap) and a constant volume of 200 μL . The flow curves were described using the power law model, appropriate for shear-thinning and shear-thickening fluids.

$$\tau = K \cdot \dot{\gamma}^n \quad (1)$$

where K is the consistency coefficient ($\text{Pa} \cdot \text{s}^n$) and the exponent n is the flow behavior index and reflects the closeness to Newtonian flow. For the special case of a Newtonian fluid ($n = 1$), the consistency coefficient K is the Newtonian viscosity of the fluid, whilst in power law fluids (shear-thinning or pseudoplastic ($0 < n < 1$) and shear-thickening fluids ($n > 1$)) apparent viscosity (η_a ; $\text{Pa} \cdot \text{s}$) will change when the shear strain rate is increased. Apparent viscosity values between different samples were compared at a shear strain rate of 60 s^{-1} .

For Oscillatory testing, a constant volume of 350 μL of sample was transferred to the rheometer equipped with a cone and plate geometry (plate, 40 mm diameter; 0.03 mm gap) and a Peltier plate set at 10°C . After 3 min of temperature stabilization, the sample was subjected to a frequency sweep from 0.1 to 100 Hz (0.1% strain, within the linear region). Viscoelastic properties were determined by monitoring the storage (G') and loss (G'') moduli as a response to the applied dynamic deformation.

3. Results and discussion

3.1. Assessment of the Maillard reaction evolution during the formation of glycoconjugates

It is well known that furosine (2-furoylmethyl-lysine), formed upon acid hydrolysis of protein-bound Amadori product of lysine, can be a useful indicator to measure the glycation extent of milk proteins (Moreno et al., 2002; Krause, Combs & Beauchemin, 2003; Fenaille, Morgan, Parisod, Tabet & Guy, 2003; Jimenez-Castaño, López-Fandiño, Olano & Villamiel, 2005a and 2005b). Figures 1A and 1C show the evolution of furosine derived from the Amadori products of the SC:Gal/Lac/DX conjugates upon storage at 50 and 60°C for 96 and 24 hours, respectively. The higher formation of furosine observed in galactosyl conjugates indicated that Gal had a higher initial rate of utilization of ϵ -amino groups of lysine residues than lactose and dextran at both temperatures. Thus, the maximum level of furosine in glycated SC:Gal system was ~ 3-fold higher than in SC:Lac system and ~ 12-fold higher than in SC:DX system at 50 and 60°C. Moreover, a noticeable decrease of furosine was also found in SC:Gal glycoconjugates after 48 hours of incubation at 50°C, indicating that the degradation of the Amadori compound prevailed over its formation.

As it was expected, the sugar reactivity towards SC was galactose > lactose > dextran. The order of reactivity according to which monosaccharides are more reactive than disaccharides and these more reactive than polysaccharides is well stated and has been previously supported in many studies (Lewis & Lea, 1950; Spark, 1969; Nacka, Chobert, Burova, Léonil, & Haertlé, 1998; Chevalier, Chobert, Mollé & Haertlé, 2001; Oliver et al., 2006c). Thus, it was observed that the smaller the carbonic chain of the sugar is, the more acyclic forms exist and the more reactive is the sugar with the amino groups of proteins. Likewise, it has been also described that DX

reacts poorly with proteins because of steric hindrance caused by the high branching degree of their molecules, which difficult the reaction of the single reducing group of each dextran molecule unavailable to react with free amino groups in the protein (Aminlari, Ramezani & Jadidi, 2005).

In good agreement with these results, the highest color development at the end of the storage period was observed in galactosyl derivatives (dark brown) followed by SC:Lac (light brown) and SC:DX (slight yellow) conjugates. As shown in figures 1B and 1D, for SC glycated with Gal at 50 and 60°C, the formation of brown compounds progressively increased over the storage period, suggesting an extensive progress of the Maillard reaction toward the most advanced stages. Lactosylated SC also showed an increase of color development over the incubation time particularly evident at 60°C, although much less marked than that of the galactosylated SC. The lowest values of browning were observed in SC glycated with DX at both temperatures, confirming a limited extent of the Maillard reaction. Such browning indices can not be attributed to the sugar caramelisation because sugars heated alone failed to generate compounds absorbing at 420 nm (data not shown). Furthermore, caramelisation mainly occurs at higher temperature and alkaline pH in aqueous solution (Laroque, Inisan, Berger, Vouland & Dufossé, 2008) and not under the relatively mild conditions assayed in this study (50-60°C at a_w 0.67 and pH 7.0).

3.2. Structural characterization of glycoconjugates

The fractions observed in the RP-HPLC profile of native SC were identified, in order of increasing retention time, as κ -casein mono-phosphorylated genetic variant A (fraction 1), κ -casein mono-phosphorylated genetic variant B and α_{S2} -casein non-phosphorylated genetic variant A (fraction 2), α_{S1} -casein octa-phosphorylated genetic variant B (fraction 3), and β -caseins penta-phosphorylated genetic variants A2 and A1 (fraction 4), and whose molecular

masses were 19,038 (fraction 1), 19,008 and 25,223 (fraction 2), 23,619 (fraction 3), and 23,987 and 24,030 Da (fraction 4), respectively, as previously reported by Léonil, Mollé, Gaucheron, Arpino, Guénot, and Maubois (1995) and van der Ven (2002) (figure 2A).

Regarding glycosylated SC, table 1 summarizes the number of molecules of Gal and Lac linked covalently to the main fractions of caseins determined experimentally by RP-HPLC/ESI-MS analyses. Deconvoluted spectra of SC : Gal and SC : Lac conjugates showed a heterogeneous mixture of various glycoform species differing in the number of carbohydrate residues linked to the peptidic chain (table 1). Similarly, Oliver, Kher, McNaughton & Augustin (2009) recently observed a similar behavior after glycation of SC with glucose in aqueous solutions. However, no evidence of glycation of SC with DX was found after deconvolution of ESI spectra, which could be attributed to both the low ionization efficiency of polysaccharides in ESI due to the low proton affinity of carbohydrates (Hsu et al., 2007) and the slow progress of Maillard reaction using DX as reducing sugar (figure 1). Jimenez-Castaño et al. (2007) needed 4 and 10 days at 0.44 a_w , 60°C and 0.65 a_w , 50°C, respectively, to block a maximum of 1.73 lysine molecules per β -lactoglobulin molecule. For reasons of simplicity, only the most abundant genetic variant of each casein was included in table 1, i.e. κ -casein genetic variant A and β -casein genetic variant A2, as no differences were found in the glycation patterns of the genetic variants corresponding to the same casein. Furthermore, the corresponding deconvoluted spectra of glycosylated α_{s2} -casein were not satisfactory due to the low ionization exhibited by fraction 2 (figure 2B). In general terms, a higher number of Gal and Lac molecules were bound to the more abundant casein components (α_{s1} - and β -casein) as compared to the κ -casein. α_{s1} -, α_{s2} -, β - and κ -caseins are present in SC in the approximate proportions 4:1:4:1, respectively. Recent data concerning casein reactivity were in good agreement with our results (Scaloni, Perillo, Franco, Fedele, Froio et al., 2002). Thus, a maximum of 4, 3 and 1 Lac molecules linked to α_{s1} -, β - and κ -caseins, respectively, could be detected after incubation of SC for 4 days at 50 °C and for 1 day at 60 °C (Table 1). As regards

SC : Gal conjugates, a maximum of 5, 4 and 3 Gal residues bound to α_{s1} -, β - and κ -caseins, respectively, were detected after incubation of SC for 2 days at 50 °C, and 6, 5 and 3 Gal residues linked to α_{s1} -, β - and κ -caseins, respectively, after storage of SC for 4 hours at 60 °C. This data confirmed the higher reactivity of Gal in comparison to Lac. In addition, taking into account the fast progress of the Maillard reaction between SC and Gal observed from the second day of incubation at 50 °C and from the eighth hour of storage at 60 °C, as it can be deduced from the high values of absorbance at 420 nm (Figures 1B and 1D), the deconvoluted spectra corresponding to the two longest storage times (3 and 4 days at 50 °C and 8 and 24 hours at 60 °C) were very complex and confusing probably due to the elevated number of products formed during the advanced stages of the Maillard reaction. Therefore, it is plausible that a higher number of Gal molecules were actually attached to SC following long storage times as α_{s1} -, β - and κ -casein contain 14, 11, and 9 lysine residues prone to participate in the Maillard reaction (Whitney, 1988; Aminlari et al., 2005).

In an attempt to shed more light on the structural changes promoted by the reaction between Gal, Lac or DX and SC, the polymerization degree of SC was investigated by means of SEC under non-denaturing conditions (table 2). Native SC eluted as two partly resolved peaks, eluting the first one very near the void volume, in agreement with several previous studies (Lynch, Mulvihill, Law, Leaver & Horne, 1997; Lucey, Srinivasan, Singh & Munro, 2000). When SC was treated with urea and DTT, the peak 1 substantially decreased with regard to peak 2 (chromatogram not shown), suggesting that the former was constituted by covalent and non-covalent casein aggregates, concretely by κ -casein polymers and some α_{s1} - and β -casein complexes (Lucey et al., 2000). Moreover, peak 2 presented a wide size distribution from $M_r \sim 80$ kDa at the start of peak, to M_r of 25-30 kDa, close to the theoretical molecular weight of casein monomers, at the trailing end of the distribution, according to Lucey et al. (2000). The control protein heated in absence of sugars showed a slight increase of the high M_w (HM_w) fraction as

compared to native SC, indicating the formation of some minor aggregated forms during heating, probably due to hydrophobic interactions. For SC:Gal conjugates at both temperatures and SC:Lac conjugates at 60°C, a notable increase of HM_w fraction with regard to low M_w (LM_w) fraction was observed with increasing incubation time, especially in galactosyl derivatives at 50°C (figure 3). This indicates that glycation of SC promoted its polymerization mainly through covalent cross-linking, produced at the most advanced stages of the Maillard reaction. SEC profiles of lactosyl derivatives at 50°C and SC:DX conjugates at 50 and 60°C were very similar to those of unglycated protein, confirming the limited extent of the Maillard reaction to the more advanced stages under these conditions, in agreement with the results derived from the furosine and browning evaluation (figure 1). The greater browning development and accelerated polymerization of SC:Gal might be due to the rapid degradation of the corresponding Amadori compound, tagatosyl-lysine, into highly reactive Maillard reaction products and brown polymers. Similar trend was previously described by Kato, Matsuda, Kato and Nakamura (1988) for the ovalbumin glycated with glucose and lactose at 50°C. These authors reported that the limited extent of the Maillard reaction in the lactosyl conjugates is attributed to the galactopyranoside group on the C-4 hydroxyl group of glucose which acts to protect the Amadori adduct from further degradation.

3.3. Effect of glycation on flow and viscoelastic properties of sodium caseinate

Figure 4 shows the consistency coefficient, K , and the flow behavior index, n , of native, control heated, control heated+carbohydrate and SC glycated with Gal, Lac and DX upon hydration at 30 mg/mL protein concentration incubated at 0.67 a_w for 96 and 24 hours at 50 and 60°C, respectively. Unlike SC:Gal/Lac systems, no consistent differences between flow properties of SC:DX conjugate and the control heated SC+DX were observed, indicating that the alteration

of the flow properties of SC glycated with DX was mainly due to the intrinsic properties of the polysaccharide. So, from now, to study the effect of the Maillard reaction on rheological properties of SC, we will focus on the caseinate glycated only with Gal and Lac.

In the shear strain rate range applied (0 to 120 s⁻¹), the flow of native and control heated SC was very close to Newtonian ($n \sim 1$), with correlation coefficients (R^2) above 0.993. However, for SC glycoconjugates, an increase of the consistency coefficient K as well as an evolution of the flow behavior to markedly pseudoplastic was observed with increasing incubation time, showing better R^2 values (> 0.997) with the power law model than with the Newtonian model. This effect was particularly notable in galactosyl derivatives upon storage at 50 and 60°C, and in lactosyl derivatives after storage at 60°C. Thus, the flow curves of glycoconjugates SC:Gal (72 hours, 50°C), SC:Gal (8 hours, 60°C), and SC:Lac (24 hours, 60°C) had a flow behavior index (n) of 0.73 (vs. 0.96 for control heated), 0.76 (vs. 0.95 for control heated) and 0.80 (vs. 0.94 for control heated), and consistency factors (K) 4.9-fold, 3.1-fold and 2.5-fold higher than that of the control heated SC, respectively. This suggests a high level of internal structure within SC glycoconjugates.

In agreement with these results, the viscosity for control SC+Gal/Lac at time 0 did not depend on the shear rate, remaining fairly constant over the range investigated and displaying, therefore, a very close Newtonian flow. In contrast, for SC:Gal and SC:Lac glycoconjugates the viscosity decreased with increasing shear rate over the range investigated (figure 5). This behavior is characteristic of pseudoplastic fluids, which are well described by a power law model. Moreover, this shear rate dependence of the viscosity was particularly evident in SC glycated with Gal at 50°C followed by SC glycated with Gal at 60°C > Lac at 60°C > Lac at 50°C. These results indicate that glycation with Gal produce more substantial alteration in flow behavior of SC, from Newtonian to markedly pseudoplastic with increasing incubation time, than glycation with Lac.

Likewise, apparent viscosity of glycated SC increased with the incubation time, whereas the apparent viscosity of control heated SC remained low during all storage. The largest increase in apparent viscosity was observed in SC glycated with Gal from 48 hours of incubation at 50°C, followed by SC:Gal and SC:Lac from 2 and 4 hours at 60°C, respectively (figure 6). Because of the greater reactivity and faster progress of the Maillard reaction with Gal, SC:Gal conjugates upon storage for 96 and 24 hours at 50 and 60°C, respectively, gave rise to dispersions containing highly insoluble brown polymers ("insoluble particles"), which interfered with measures of rheometer, being impossible to determine their viscosity. These results are in agreement with those of Oliver et al. (2006b) who glycated SC with glucose, ribose, fructose, lactose and fructo-oligosaccharide (inulin). However, these authors did not investigate the viscoelasticity of such glycoconjugates, which would be needed to completely describe their flow behavior, since they have both properties of solids and liquids.

Viscoelastic properties of glycoconjugates were determined upon hydration (at 150 mg/mL protein concentration) by means of oscillatory tests, monitoring the storage (G') and loss (G'') moduli as a response to the applied dynamic deformation. Figure 7 depicts the dependence of the G' modulus on frequency and incubation time for the glycoconjugates. The G' of native and control heated SC at 50 and 60°C remained low and constant over the storage period ($G' < 0.1$). In contrast, G' of glycoconjugates showed a general trend to increase with increasing incubation time, i.e. with the progress of the Maillard reaction. At longer incubation times, some glycoconjugates showed a gel-like behaviour. Frequency sweeps confirmed gelled samples when the G' was > 1 and greater than the loss modulus (G''), the G' and G'' were parallel over the frequency range. This behavior seemed to be clear in galactosyl derivatives from 48 and 8 hours of storage at 50 and 60°C, respectively). Lactosyl derivatives also formed gels from 8 hours at 60°C. For these conjugates with gel-like behavior G' did not show a strong dependence on frequency, displaying a slight increase at higher frequency (> 50 rad/s), what is suggestive of a

well-organised and cohesive structure (true gel). G' of SC:Gal conjugates was higher than that of SC:Lac conjugates. Moreover, increasing frequency had less effect on gels with galactose than with lactose. This suggested that the gels containing galactose formed more elastic networks than the gels containing lactose. In SC:Lac systems upon storage for 72 and 96 hours at 50°C, the G' was also greater than the G'' fairly over the frequency range; however we did not consider them as gelled systems since G' was < 1 . In these cases, G' and G'' increased over the frequency range studied, but at higher frequency, G' modulus slightly dropped, most probably due to rheometer inertial effects. These results confirm a significantly higher level of internal structure in SC glycosylated with Gal than with Lac, in agreement with the results of flow properties determination.

At shorter storage times, the viscoelastic behavior of glycoconjugates was characterized by a G'' greater than G' over the frequency range. Likewise, the G' remained low and fairly constant, ~ 0 ($1 > G' > 0$), over the frequency range (figure 7). These glycoconjugates displayed, therefore, a viscous-like flow characteristic of Newtonian or slightly pseudoplastic fluids.

A number of studies carried out with proteins from different origin, mainly globular proteins, support that protein aggregation and cross-linking associated with the advanced stages of the Maillard reaction have an important impact on the rheological properties of these proteins (Hill et al., 1992; Armstrong et al., 1994; Cabodevila et al., 1994; Oliver et al., 2006b). According to these authors, the alteration of flow and viscoelastic properties of SC glycosylated, particularly with galactose, observed in this work could be probably due to a caseinate polymerization through covalent cross-links induced by the advanced Maillard reaction.

4. Conclusions

Rheological changes associated with SC glycated via the Maillard reaction were found to be influenced by the sugar molecular weight and reaction temperature. Due to the limited extent of the Maillard reaction observed in SC when was glycated with DX at 50 and 60°C, the effect of DX on rheological properties of SC could be mainly attributed to the intrinsic properties of the polysaccharide. In contrast, glycation with Gal and Lac at both temperatures altered the SC flow characteristics with increasing storage time, so that SC glycoconjugates showed a higher viscosity and more pseudoplastic flow behavior than native and control heated SC. Likewise, these Maillard complexes were characterised by intense viscoelastic character. For glycated SC, oscillatory testing showed an increasing G' modulus with incubation time, so that samples exhibited a gradation in flow behavior from highly liquid of unmodified SC to viscous-like of glycoconjugates for shorter incubation times and to gelled network of glycoconjugates for longer incubation times. Such results suggest a great level of internal structure within SC glycoconjugates. The most remarkable effects of glycation on protein flow and viscoelastic properties were observed in galactosyl conjugates incubated at 50°C, followed by SC:Gal 60°C > SC:Lac 60°C > SC:Lac 50°C, in agreement with the results derived from the assessment of the Maillard reaction evolution and structural characterization of glycoconjugates. These showed a higher reactivity of Gal during the initial stages of the Maillard reaction, as well as a greater browning development and an accelerated polymerization of SC glycated with Gal as compared to Lac and DX, suggesting that such improvement in rheological properties of glycated SC could be attributed to protein aggregation through covalent cross-linking, produced at the most advanced stages of the Maillard reaction.

Taken together, results of this study show that by controlling the rate and extent of the Maillard reaction, it is possible to increase the viscosity and gelling properties of SC in a technologically feasible and 'food-safe' way without the addition of toxic reagents. The improvement of rheological characteristics of SC could produce an equivalent functionality with a

smaller amount of added protein, helping to reduce production costs, as well as to promote its use as food ingredient with added value to develop customer-favored products with a competitive edge in the marketplace.

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581

FIGURE CAPTIONS

Figure 1. Evolution of the content of furosine derived from the Amadori products (**A** and **C**) and absorbance at 420 nm (**B** and **D**) during storage at 0.67 a_w upon 96 hours at 50°C (**A** and **B**) and 24 hours at 60°C (**C** and **D**) of the Maillard conjugates: —◆— SC:Gal; —✕— SC:Lac; —■— SC:DX. Data points represent the mean of duplicate determinations with error bars indicating the standard deviations.

Figure 2. UV- (**A**) and total ion current- (TIC) (**B**) chromatograms of native SC following LC/ESI-MS analysis.

Figure 3. SEC elution profile of SC glycated with Gal at 50°C for 96 hours ($a_w=0.67$) using a Superdex 75 column on a FPLC system under non-denaturing conditions.

Figure 4. Changes in consistency factor K (Pa s^n) (fluid consistency) and flow index (n) of — control heated, control heated + sugar (—◆— SC+Gal; —✕— SC+Lac; —□— SC+DX) and glycated SC (—◆— SC:Gal; —✕— SC:Lac; —■— SC:DX), at 30 mg/mL of SC, incubated at 0.67 a_w for 96 hours at 50°C (**A** and **C**) and for 24 hours at 60°C (**B** and **D**). Data points represent the mean of duplicate determinations, being the standard deviation in all cases < 0.01 .

Figure 5. Shear rate dependence of the apparent viscosity of SC:Gal/Lac glycoconjugates (at 30 mg/mL of SC) upon storage for 4 days at 50°C and 1 day at 60°C.

Figure 6. Effect of glycation via the Maillard reaction on apparent viscosity of SC glycated with Gal and Lac (at 30 mg/mL of SC) at 50 (**A**) and 60°C (**B**).

607

608 **Figure 7.** Dependence of the storage modulus (G') on frequency and incubation time for
609 control heated SC and and SC:Gal/Lac conjugates (at 150 mg/mL of SC) incubated for 96 hours
610 at 50°C (**A**) and 24 hours at 60°C (**B**).

Figure 1

Figure 1. Corzo-Martinez et al.

Figure 2

Figure 2. *Corzo-Martinez et al.*

Figure 3

Figure 3. *Corzo-Martinez et al.*

Figure 4

Figure 4. Corzo-Martinez et al.

Figure 5

Figure 5. *Corzo-Martinez et al.*

Figure 6. *Corzo-Martinez et al.*

Figure 7

Figure 7. Corzo-Martinez et al.

A

B

Table 1. Experimentally determined molecular masses of the different forms of κ -, α_{s1} - and β -casein and assigned number of galactose (Gal) and lactose (Lac) molecules bound to sodium caseinate (SC) derived from the LC-ESI mass spectrometry analyses.

Temperature	Sample	Storage time (hours)	Observed molecular masses of κ -casein A ^a (no. of linked sugar adducts)	Observed molecular masses of α_{s1} -casein B ^b (no. of linked sugar adducts)	Observed molecular masses of β -casein A2 ^c (no. of linked sugar adducts)
50°C	SC:Gal	4	19,038 (0 Gal)	23,619 (0 Gal)	23,987 (0 Gal)
			19,200 (1 Gal)	23,781 (1 Gal)	24,149 (1 Gal)
			19,362 (2 Gal)	23,943 (2 Gal)	24,311 (2 Gal)
				24,105 (3 Gal)	
				24,267 (4 Gal)	
	SC:Lac	4	19,038 (0 Lac)	23,619 (0 Lac)	23,987 (0 Lac)
			19,362 (1 Lac)	23,943 (1 Lac)	24,311 (1 Lac)
				24,267 (2 Lac)	24,635 (2 Lac)
	SC:Gal	48	19,038 (0 Gal)	23,619 (0 Gal)	23,987 (0 Gal)
			19,200 (1 Gal)	23,781 (1 Gal)	24,149 (1 Gal)
			19,362 (2 Gal)	23,943 (2 Gal)	24,311 (2 Gal)
			19,524 (3 Gal)	24,105 (3 Gal)	24,473 (3Gal)
				24,267 (4 Gal)	24,635 (4 Gal)
				24,429 (5 Gal)	
60°C	SC:Lac	4	19,038 (0 Lac)	23,619 (0 Lac)	23,987 (0 Lac)
			19,362 (1 Lac)	23,943 (1 Lac)	24,311 (1 Lac)
				24,267 (2 Lac)	24,635 (2 Lac)
	SC:Gal	48	19,038 (0 Lac)	23,619 (0 Lac)	23,987 (0 Lac)
			19,362 (1 Lac)	23,943 (1 Lac)	24,311 (1 Lac)
				24,267 (2 Lac)	24,635 (2 Lac)
				24,591 (3 Lac)	
	SC:Lac	72	19,038 (0 Lac)	23,619 (0 Lac)	23,987 (0 Lac)
			19,362 (1 Lac)	23,943 (1 Lac)	24,311 (1 Lac)
				24,267 (2 Lac)	24,635 (2 Lac)
				24,591 (3 Lac)	
	SC:Gal	96	19,038 (0 Lac)	23,619 (0 Lac)	23,987 (0 Lac)
			19,362 (1 Lac)	23,943 (1 Lac)	24,311 (1 Lac)
				24,267 (2 Lac)	24,635 (2 Lac)
				24,591 (3 Lac)	24,959 (3 Lac)
				24,915 (4 Lac)	
60°C	SC:Gal	2	19,038 (0 Gal)	23,619 (0 Gal)	23,987 (0 Gal)
			19,200 (1 Gal)	23,781 (1 Gal)	24,149 (1 Gal)
			19,362 (2 Gal)	23,943 (2 Gal)	24,311 (2 Gal)
				24,105 (3 Gal)	24,473 (3Gal)
				24,267 (4 Gal)	24,635 (4 Gal)
				24,429 (5 Gal)	
	SC:Lac	4	19,038 (0 Gal)	23,619 (0 Gal)	23,987 (0 Gal)
			19,200 (1 Gal)	23,781 (1 Gal)	24,149 (1 Gal)
			19,362 (2 Gal)	23,943 (2 Gal)	24,311 (2 Gal)
			19,524 (3 Gal)	24,105 (3 Gal)	24,473 (3Gal)
				24,267 (4 Gal)	24,635 (4 Gal)
				24,429 (5 Gal)	24,797 (5 Gal)
				24,591 (6 Gal)	

SC:Lac	2	19,038 (0 Lac)	23,619 (0 Lac)	23,987 (0 Lac)
		19,362 (1 Lac)	23,943 (1 Lac)	24,311 (1 Lac)
			24,267 (2 Lac)	24,635 (2 Lac)
	4	19,038 (0 Lac)	23,619 (0 Lac)	23,987 (0 Lac)
		19,362 (1 Lac)	23,943 (1 Lac)	24,311 (1 Lac)
			24,267 (2 Lac)	24,635 (2 Lac)
	8	19,038 (0 Lac)	23,619 (0 Lac)	23,987 (0 Lac)
		19,362 (1 Lac)	23,943 (1 Lac)	24,311 (1 Lac)
			24,267 (2 Lac)	24,635 (2 Lac)
			24,591 (3 Lac)	
	24	19,038 (0 Lac)	23,619 (0 Lac)	23,987 (0 Lac)
		19,362 (1 Lac)	23,943 (1 Lac)	24,311 (1 Lac)
			24,267 (2 Lac)	24,635 (2 Lac)
			24,591 (3 Lac)	24,959 (3 Lac)
			24,915 (4 Lac)	

^a Mono-phosphorylated.

^b Octo-phosphorylated.

^c Penta-phosphorylated.

Table 2. High M_w and Low M_w fractions of native, control heated and glycosylated sodium caseinate (SC) with galactose (Gal), lactose (Lac) and dextran (DX) incubated at 50°C for 96 hours and 60°C for 24 hours, estimated after their analysis by size exclusion chromatography under nondenaturing conditions.

Temperature	Sample	Storage time (hours)	HM _w fraction (%)	LM _w fraction (%)	HM _w / LM _w ratio
50°C	Native SC	0	23.99 ± 0.28	76.01 ± 0.28	0.31
	Heated SC	4	24.39 ± 0.41	75.61 ± 0.41	0.32
		48	30.71 ± 0.84	69.29 ± 0.84	0.44
		72	30.89 ± 0.33	69.11 ± 0.33	0.45
		96	32.17 ± 1.13	67.85 ± 1.13	0.47
	SC:Gal	4	25.02 ± 0.65	74.98 ± 0.65	0.33
		48	53.60 ± 0.85	46.40 ± 0.85	1.16
		72	76.49 ± 0.49	23.51 ± 0.49	3.25
		96	81.25 ± 0.71	18.75 ± 0.71	4.33
	SC:Lac	4	24.58 ± 1.56	75.42 ± 1.56	0.33
		48	33.35 ± 1.02	66.65 ± 1.02	0.50
		72	35.13 ± 0.36	64.87 ± 0.36	0.54
		96	37.22 ± 0.44	62.78 ± 0.44	0.59
	SC:DX	96	33.32 ± 0.23	66.68 ± 0.23	0.50
60°C	Heated SC	2	23.82 ± 0.76	76.18 ± 0.76	0.31
		4	26.81 ± 0.88	73.19 ± 0.88	0.36
		8	27.05 ± 1.41	72.95 ± 1.41	0.37
		24	30.59 ± 0.94	69.41 ± 0.94	0.44
	SC:Gal	2	25.97 ± 0.76	74.03 ± 0.76	0.35
		4	33.13 ± 0.84	66.87 ± 0.84	0.50
		8	39.82 ± 0.54	60.18 ± 0.54	0.66
		24	78.40 ± 0.35	21.60 ± 0.35	3.63
	SC:Lac	2	24.44 ± 1.12	75.56 ± 1.12	0.32
		4	27.85 ± 0.89	72.15 ± 0.89	0.39
		8	36.52 ± 0.99	63.48 ± 0.99	0.57
		24	39.82 ± 1.70	60.18 ± 1.70	0.66
	SC:DX	24	33.90 ± 1.05	66.10 ± 1.05	0.51