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Gel mechanical properties of milk whey protein–dextran conjugates obtained by Maillard reaction

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ABSTRACT

The effect of Maillard reaction on the mechanical properties of whey protein isolate (WPI) heat-induced gels was evaluated. WPI and dextran (15–25 kDa) conjugates were obtained by controlled dry heating during storage at 60 °C and 63% relative humidity for 2, 5 and 9 days. Changes in browning intensity and content of free amino groups were used to estimate the Maillard reaction. A decrease in free amino groups of WPI was observed when increasing polysaccharide concentration and reaction time. An increase in both a^* and b^* CIE Lab colour parameters indicated the development of a reddish-brown colour, typical of the Maillard reaction. Uniaxial compression and stress relaxation tests were performed to measure the mechanical properties of mixed and conjugate gels. Maillard reaction significantly modified the mechanical properties of WPI/DX gels, and even prevented fracture when conjugate gels were subjected to 80% deformation in uniaxial compression test.

1. Introduction

Proteins offer an extensive range of desirable functional properties for use in the food industry, as well as in pharmaceuticals and cosmetics. Whey proteins, in particular, which are a by-product of cheese whey industry, are widely used because of their technological applications and nutritional value (Fitzsimons, Mulvihill, & Morris, 2008; Qi & Onwulata, 2011). They represent 20% of total milk proteins and are composed of β-lactoglobulin (β-lg) (50%), α-lactalbumin (α-lc) (20%), serum albumin, immunoglobulin and minor proteins (Cayot & Lorient, 1997). These proteins can form thermal gels, which are stabilized by new intra and interchain disulfide bonds (β-lg has two disulfide bonds and one free sulfhydryl group and α-lc has four disulfide bonds) formed in the denaturation–aggregation processes occurring during gelation. Although gelation of whey proteins is mostly achieved by heating (Baeza, Gugliotta, & Pilosof, 2003; Baeza & Pilosof, 2001; Bertrand & Turgeon, 2007; Bryant & Mc Clements, 2000; Spotti, Santiago, Rubiolo, & Carrara, 2012), it can also be achieved by cold gelation process (van den Berg, van Vliet, van der Linden, van Boekel, & van de Velde, 2007a, 2007b) or by high pressure treatment (Keim & Hinrichs, 2004). There are many studies about gelation as well as about mechanical and rheological properties of whey protein solutions and gels. In these works, whey proteins are often mixed with polysaccharides. The combination of native or denaturated proteins with neutral or anionic polysaccharides can give a great number of different structures which can be regarded as highly relevant models of food structures (van den Berg et al., 2007a, 2007b; de Jong & van de Velde, 2007; Tolstoguzov, 2006). The effects of polysaccharides on the aggregation and the gel formation of a whey protein solution differ depending on the nature of the polysaccharide, pH, ionic strength, temperature and concentrations used (van den Berg et al., 2007a). Gelation of mixed biopolymer solutions can result in three types of arrangement: interpenetrating, coupled, and phase-separated structures (Morris, 1986). Interpenetrating networks consist of two independent network structures which interact only through mutual entanglements (van den Berg et al., 2007b). Coupled networks are formed when there is an attractive interaction between the two biopolymers resulting in a single network structure. Phase-separated networks are formed by incompatible biopolymers where interactions between the different polymers are repulsive and/or when the two types of polymers show varying affinity towards the solvent (van den Berg et al., 2007b; Grinberg & Tolstoguzov, 1997; Tolstoguzov, 1997). Phase-separated structures are the most likely outcome of gelation (Spotti et al., 2012; Tavares, Monteiro, Moreno, & Lopes da Silva, 2005).

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The functionality of proteins is governed by their physicochemical and intrinsic structural properties. Functional properties of proteins can be modified through chemical, physical or enzymatic methods such as acidification, heating, hydrolysis, acetylation, esterification, amidation, and enzymatic cross-linking, among others (Abtahi & Aminlari, 1997; Nagasawa, Takahashi, & Hattori, 1996; Nakamura, Kato, & Kobayashi, 1992). These treatments modify ability to form gels, water retention, solubility, foaming and emulsifying capacity, etc (Blecker et al., 1997). Most of the chemical methods have not been used for food applications because of their potential health hazard, such as the occurrence of harmful products, changes in digestibility and/or allergenicity (Kato, 2002) and occasionally, if the presence of toxic reagents is required, their use as a food ingredient will be limited (Hattori, Aiba, Nagasawa, & Takahashi, 1996; Nagasawa et al., 1996).

Interaction with polysaccharides has been reported as a method of modifying functional properties of proteins (Jimenez-Castaño, Villamiel, & Lopez-Fandiño, 2007). Several types of intermolecular interactions can be produced in a protein–polysaccharide system: hydrophobic and electrostatic interactions, hydrogen and covalent bonds. One example of naturally occurring covalent bonds between proteins and polysaccharides is the Maillard reaction.

The Maillard reaction has been extensively studied from nutritional, toxicological and sensory aspects, due to the development of flavours and colours that appear as a result of food processing (Goloberg, Weijng, & Melpomeni, 2004; Miller & Gerrard, 2005). The mechanisms of this reaction are complex. First, condensation of a protonated amino group of a protein with a carbonyl group of a reducing sugar (under certain conditions of temperature and water activity) forms a Schiff base. Then, a ketosamine is formed, which is more stable and it is known as the Amadori product (Miller & Gerrard, 2005). Subsequently, a wide variety of reactions can occur, including polymerizations, cyclizations, etc., to form a wide range of compounds, such as furan, nitrogenous heterocyclic products and insoluble brown polymeric compounds called melanoidins. The last compounds are responsible for the typical reddish-brown colour of Maillard reaction products (Ordoñez Perda et al., 1998).

The study of this reaction in controlled synthesis of protein–polysaccharide conjugates to generate new compounds with improved functional properties started some years ago. Properties of protein–polysaccharide conjugates obtained via Maillard reaction depend on both protein conformation and polysaccharide characteristics such as hydrophobicity, viscosity, chain length and number of joints (Jimenez-Castaño et al., 2007; Kato, Sasaki, Furuta, & Kobayashi, 1990). It was shown that protein–polysaccharide conjugates had better emulsifying properties (Einhorn-Stoll, Ulbrich, Sever, & Kunzek, 2005; Oliver, Melton, & Stanley, 2006), foaming properties (Dickinson & Izgi, 1996), protein solubility (Katayama, Shima, & Saeki, 2002) and heat stability (Chevalier, Chobert, Genot, & Haertlé, 2001) than their precursor proteins.

There are few studies focussing on the influence of the Maillard reaction on gelation of protein/polysaccharide conjugate systems, especially on their effect on gel mechanical properties. Accordingly, the objective of this work was to study the mechanical properties of WPI/dextran mixed gels as compared with WPI/dextran conjugate gels obtained by Maillard reaction and the effect of reaction time on these properties.

Dextrans are polysaccharides composed mainly of a linear chain of α (1,6) D glucosyl residues. They are widely used for conjugating proteins because their neutral nature prevents the electrostatic complex formation. Besides the dextran structure is very flexible in aqueous solution, making it unable to form a gel, and this therefore suitable for studies of protein gelation.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) (BIPRO™) was kindly provided by Davisco Foods International Inc. (Minnesota, USA). Its percent composition was 97.9% (dry basis) protein, 0.2% fat, 1.9% ash and 4.8% moisture. Dextran (DX) of 15–25 kDa molecular weight was obtained from Sigma–Aldrich Co. (St. Louis, USA).

2.2. Obtaining WPI/DX mixed and conjugates systems

WPI/DX mixed solutions were obtained at a constant protein concentration of 12% w/w while DX concentration ranged from 3.6 to 10.8% w/w. A 12% w/w WPI solution without DX was used as control in all reaction times. Sodium azide (0.2% w/w) was used as bactericide and pH was adjusted to pH 7.0 with 0.01 M NaOH or 0.01 M HCl. WPI/DX solutions were lyophilized, and the powders obtained were incubated for 2, 5 or 9 days at 60 °C and 63% relative humidity. All samples were stored at −18 °C until use. The powders were dissolved with ultrapure water to their original concentration (12% w/w WPI − 3.6–10.8% w/w DX) to obtain WPI/DX conjugate solutions, 24 h before use.

Mixed solutions were prepared by mixing WPI, DX and ultrapure water at equal concentrations to those of conjugate solutions (12% w/w WPI − 3.6–10.8% w/w DX). These solutions were prepared at least 24 h before use. Both solutions were maintained at 4 °C until use.

2.3. Colour measurement of the conjugate powders

A model 508D/8° 8 mm Minolta colorimeter (Tokyo, Japan) was used for the experience. The CIE Lab system, defined in rectangular coordinates (L^*, a^*, b^*), with a 65° illuminant and 10° observer angle was applied. The following parameters: L^* (lightness), a^* (+red, −green) and b^* (+yellow, −blue) were evaluated for conjugate powders. All samples were sieved through an ASTM 40 mesh (420 μm) before observations. Lyophilized WPI (without incubation) was used as control.

2.4. Free amino groups detection using O-phthaldialdehyde (OPA) technique

The degree of modification of the primary amino groups was determined indirectly by the specific reaction between OPA and free primary amino groups in proteins (Sun et al., 2011). WPI/DX conjugate solutions (Section 2.2) were diluted to 4% w/w. To 200 μl of these solutions, 800 μl of 0.1 M sodium tetraborate buffer solution, 100 μl of 20% (w/v) SDS and 100 μl 2-mercaptoethanol were added. Then, the samples were immersed in a water bath at 90 °C for 5 min. OPA reagent was prepared as follows: 160 mg OPA was dissolved in 4 ml of absolute ethanol, 10 ml 20% (w/v) SDS, 100 ml 0.1 M sodium tetraborate buffer solution (containing 400 μl 2-mercaptoethanol) and then made up to 200 ml with deionized water. To 100 μl of sample solution, 2 ml of OPA reagent was added. Absorbances were measured at 340 nm with a Lambda 20 spectrophotometer (Perkin–Elmer, USA). To calculate the decrease in free amino groups (FAG) of conjugate samples, WPI without incubation was used as control and reference (100%), applying the following equation:

$$\%\,\mathrm{FAG} = A_S/A_{WPI}{}^{*}100 \quad (1)$$

where A_S is the absorbance of the sample and A_{WPI} the absorbance of WPI control.

2.5. Preparation of WPI/DX mixed and conjugate heat induced gels

Gels were prepared in lubricated glass cylinders (50 mm length; 15 mm internal diameter) closed at both ends with rubber stoppers. After filling with solution (mixed or conjugate; Section 2.2) the cylinders were placed in a water bath at 80 °C for 30 min to obtain heat-induced gels, which were then stored for 24 h at 4 °C before rheological measurements were made.

2.6. Uniaxial compression and stress relaxation tests

Uniaxial compression and stress relaxation tests were carried out with a universal testing machine (model 3344, INSTRON Corp., Norwood, USA) using two parallel plates (diameter: 60 mm) lubricated with a thin layer of paraffin oil to minimize friction. The samples were cylinders 15 mm in length and 15 mm in diameter. Both tests were performed in a room with controlled temperature (22 °C) at a crosshead speed of 1 mm/s (de Jong & van de Velde, 2007; Ribeiro, Rodriguez, Sabadini & Cunha, 2004; Yamamoto & Cunha, 2007). As regards uniaxial compression test, gels were compressed to 80% of their initial height.

Height and force data were transformed as follows: True or Hencky stress, σ_H, can be defined as (Steffe, 1992; Yamamoto & Cunha, 2007):

$$\sigma_H = F(t)\cdot H(t)/(H_0\cdot A_0) \quad (2)$$

Similarly, the Hencky strain, ε_H, was calculated as:

$$\varepsilon_H = \ln(H(t)/H_0) \quad (3)$$

where $F(t)$ and $H(t)$ are the force and the height at a given time t, and A_0 and H_0 are the initial area and height of the gel, respectively (Steffe, 1992; Yamamoto & Cunha, 2007).

Parameters calculated from uniaxial compression data were: maximum stress (σ_M), which is the maximum value of σ_H until rupture, calculated from Equation (2); maximum strain (ε_M), which is the maximum value until rupture, calculated with Equation (3); W_F, which is the work of fracture associated with the hardness, calculated as the area under the curve σ_H vs ε_H between 0 and ε_M; Young's modulus (E), which is calculated as the slope of the linear and initial region of σ_H vs ε_H curve (5% strain) (Steffe, 1992); and rupture deformation (Rup. Def (%)), which is the gel deformation calculated as follows:

$$(\text{final height} - \text{initial height})/\text{initial height} \times 100 \quad (4)$$

On the other hand, in stress relaxation test, the sample was compressed 5% (with respect to the initial height of the gel) and then stress versus time was recorded during 600 s. The deformation used (5%) was found to be within the linear viscoelastic region (data not shown).

Hencky stress (σ_H from Equation (1)) vs time curves were obtained from the stress relaxation data. The maximum stress relaxation modulus (G_0) was calculated as:

$$G_0 = \sigma_M/\gamma_{const} \quad (5)$$

where σ_M is maximum stress calculated with Equation (2) and γ_{const} is the dimensionless strain applied in the test (0.05 in this case) (Steffe, 1992).

2.7. Confocal scanning laser microscopy

An inverted Model TE-2000-E2 Nikon Eclipse microscope (Japan), motorized with optical DIC/Nomarski and infinity corrected optics was used. Protein solutions were non-covalently stained with 10 $\mu l/g_{prot}$ of Rhodamine B solution at 1 mg/ml (Spotti et al., 2012). The required amount of Rhodamine B was mixed with biopolymer solutions before gelation. Then, the coloured solutions were placed in glass cylinders with rubber stoppers. The cylinders were placed in a water bath at 80 °C for 30 min, and then at 4 °C for at least 24 h before observations. Observations of whey proteins were made by excitation of Rhodamine B at 544 nm, the emission being recorded between 550 and 750 nm. A 40× objective and a zoom of 10 were used in all the samples. Each image was composed of 1024 × 1024 pixels with a field of 63.65 × 63.65 μm.

2.8. Statistical analysis

All measurements were performed at least in triplicate. Mean values and their corresponding standard error were calculated and presented in graphs as coordinate pairs with their corresponding error bar. For statistical treatment of data, *StatGraphics Centurion XV* software was used and analysis of variances (ANOVA) was done. When statistical differences were found, Duncan's test ($\alpha = 0.05$) was carried out. Analysis and graphic presentations were performed using *OriginPro 7.5 SR0* software (OriginLab Corporation, Northampton, USA).

3. Results and discussion

3.1. Colour development of WPI/DX conjugate systems

The reddish brown colour occurrence is a clear indicator of Maillard reaction (MR) progress. It is known that polysaccharides and proteins form coloured compounds in advanced stages of MR. Colour development was measured by CIE Lab parameters, the results being shown in Table 1. WPI lyophilized and incubated for 2, 5 and 9 days had no colour development when compared with WPI lyophilized without incubation (used as control), as indicated by a^*, b^* and L^* CIE Lab parameters. In WPI/DX conjugate systems, an increase in a^* (+red) and b^* (+yellow) parameters were found with increasing DX concentration and reaction time. This increase was most pronounced for the WPI/DX conjugate system incubated for 9 days. Lightening, indicated by L^* parameter, decreased with an increment of DX concentration and reaction time due to the increase in colour powders, indicated by a^* and b^* parameters. The reddish brown colour shown by WPI/DX systems is an indicator of

Table 1
CIE Lab colour parameters for lyophilized WPI without incubation (0 days) and WPI/dextran conjugate powders incubated for 2, 5 and 9 days. All concentrations tested.

Days	DX conc (% w/w)	a^*	b^*	L^*
0	0	0.30 ± 0.015	11.0 ± 0.1	88.9 ± 0.0
2	0	−0.25 ± 0.01^{aA}	12.0 ± 0.0^{aA}	89.3 ± 0.0^{aA}
	3.6	3.85 ± 0.05^{bA}	22.8 ± 0.1^{bA}	84.7 ± 0.3^{bA}
	7.2	3.94 ± 0.01^{cA}	22.5 ± 0.0^{bA}	84.5 ± 0.1^{bA}
	10.8	4.18 ± 0.02^{dA}	24.3 ± 0.0^{cA}	84.7 ± 0.0^{bA}
5	0	−0.02 ± 0.01^{aB}	11.7 ± 0.0^{aB}	90.6 ± 0.1^{aB}
	3.6	4.87 ± 0.09^{bB}	28.3 ± 0.2^{bB}	81.9 ± 0.3^{bB}
	7.2	4.19 ± 0.02^{cB}	24.3 ± 0.1^{cB}	84.7 ± 0.0^{cA}
	10.8	4.09 ± 0.01^{cA}	24.1 ± 0.1^{cB}	84.5 ± 0.0^{cA}
9	0	−0.07 ± 0.01^{aB}	13.3 ± 0.0^{aC}	89.9 ± 0.0^{aC}
	3.6	5.66 ± 0.02^{bC}	27.1 ± 0.0^{bC}	82.0 ± 0.1^{bB}
	7.2	8.97 ± 0.06^{cB}	31.0 ± 0.2^{cB}	76.1 ± 0.2^{cB}
	10.8	10.09 ± 0.08^{dC}	32.5 ± 0.1^{dC}	73.6 ± 0.2^{dB}

Conc. = Concentration. Values with the same letter did not show differences with Duncan's test with $\alpha = 0.05$. Lower case indicates significant differences among concentrations in the same day. Capital letter indicates significant differences among days at the same concentration.

Maillard reaction progress, since it was the presence of both dextran and WPI protein that was responsible for colour development (WPI alone with and without incubation did not show any colour).

Colour development was also observed in WPI/DX conjugate gels (Fig. 1). They showed the same trend as their conjugate powders (the intensity of brown colour increased with increasing DX concentration and reaction time). It can be seen that, besides being coloured, gels are also translucent.

3.2. Determination of free amino groups

The determination of free amino groups is commonly used to estimate the progress of MR, since it occurs by covalent attachment of carbonyl group in reducing sugars with free amino groups in proteins to form Schiff base (Wooster & Augustin, 2007). To investigate the degree of reaction progress, free amino groups of WPI (in all conditions studied) were measured using the OPA method (Fig. 2). The content of free amino groups decreased with increasing DX concentration and reaction time. Since the higher the DX concentration, the greater the number of available carboxyl groups available to react with proteins, the reaction is expected to have further progress and therefore less free amino groups. On the other hand, if reaction time is considered, WPI/DX conjugate systems had fewer free amino groups when reaction time was longer (9 days). These results are to be expected considering that more reaction progress is produced at longer times. Similar behaviour was observed by Wooster and Augustin (2007). It is important to consider that control WPI was compared with the WPI lyophilized and incubated for 2, 5 or 9 days, no significant differences being found among them. This suggests that the treatment applied (2, 5 or 9 days at 60 °C) to the different systems did not alter the availability of WPI amino groups, as did the presence of polysaccharide.

The decrease in free amino group content was coincident with the increase in browning intensity, indicating that WPI was successfully linked with dextran under the conditions studied for all reaction times.

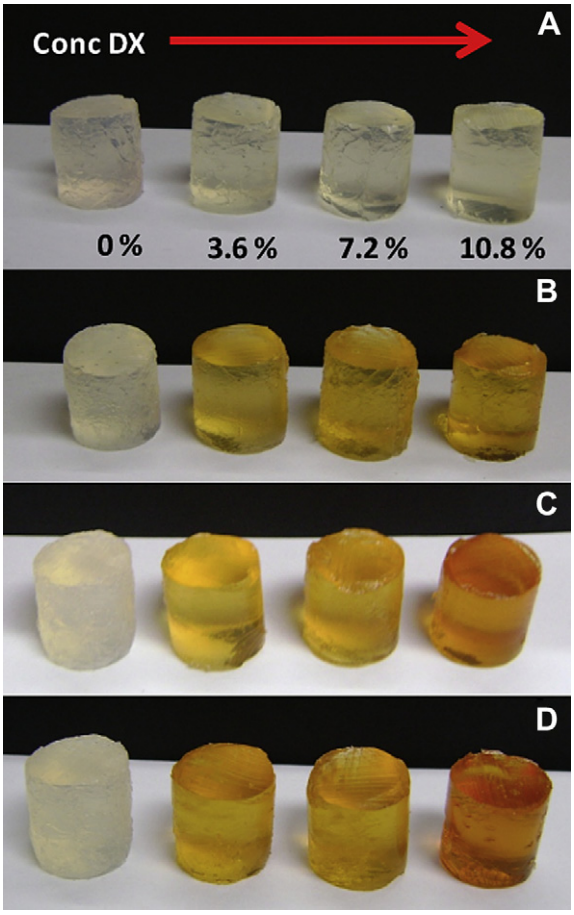

Fig. 1. Images of WPI/DX mixed gels (A) and WPI/DX conjugate gels after 2 (B), 5 (C) and 9 (D) days of Maillard reaction. Dextran concentration increases from left to right (from 0 to 10.8% w/w).

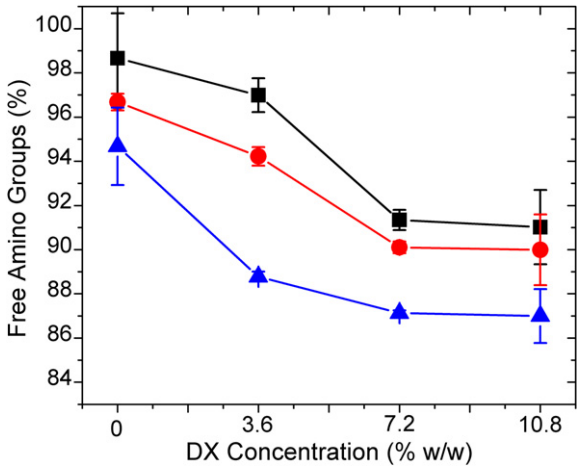

Fig. 2. Free amino groups content of WPI/DX conjugate systems versus dextran (15–25 kDa) concentration incubated for: 2 days (■), 5 days (●), and 9 days (▲) (100% of free amino groups = WPI control (WPI without incubation)).

3.3. Mechanical parameters of mixed gels

Stress vs strain curves from uniaxial compression of mixed and conjugate gels incubated for different reaction times are shown in Fig. 3. Dissimilar behaviour was found between WPI/DX mixed and conjugate gels. Whereas WPI/DX mixed gels exhibited fracture when subjected to an 80% deformation (Fig. 3A), no such behaviour was found in WPI/DX conjugate gels (Fig. 3B).

Young's modulus of WPI/DX mixed and conjugate gels can be observed in Fig. 4. Other mechanical parameters obtained from stress vs strain data for mixed gels are presented in Table 2. For mixed gels, Young's modulus (E) (Fig. 4A) and the maximum stress at fracture (σ_M) (Table 2) increased with increasing DX concentration. Work at fracture (W_F), which is related to maximum stress at fracture (σ_M), also increased with DX concentration. Maximum Hencky strain (ε_M), on the other hand, did not have significant differences for different DX concentrations. These parameters (E, σ_M and W_F) demonstrate an increase in gel hardness with increasing polysaccharide concentration. Since DX alone is unable to gel, WPI/DX mixed gels would be stabilized by the same molecular interactions involved in the formation of heat-induced whey protein gels. These interactions are mainly intra- and inter-disulfide bonds between β-lactoglobulin molecules, but probably hydrogen bonds, hydrophobic and electrostatic interactions between protein chains also play an important role in WPI gel structure.

On the other hand, in a system where there are macromolecules of different type, shape and structure, microsegregative phase separation can occur (a protein-rich and a polysaccharide-rich phase, respectively) (Tolstoguzov, 1997). This probably results in the creation of microdomains where the protein concentration is higher, and therefore, there is an increase in gel hardness because

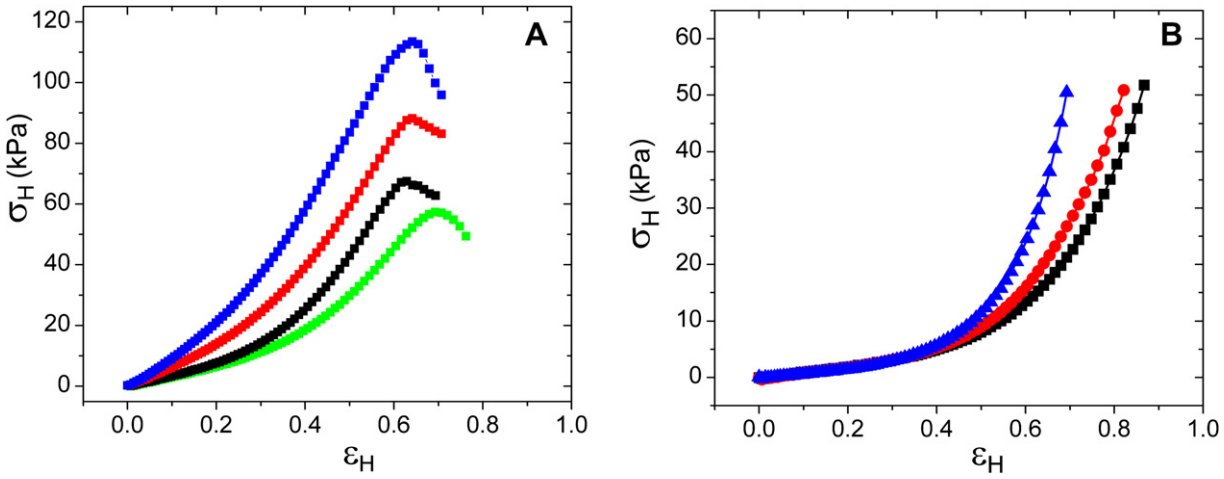

Fig. 3. Stress vs Hencky's strain for WPI/DX mixed gels of 12% w/w WPI with 0% w/w (■), 3.6% w/w (●), 7.2% w/w (▲). And 10.8% w/w DX (★) (A); and for WPI/DX conjugate gels (12% w/w WPI; 7.2% w/w DX) from dry blends incubated for 2 days (■), 5 days (●) and 9 days (▲) (B).

more network connections can be established between them. Large enhancements can be obtained by mixing a gelling polymer and a non-gelling polymer at concentrations where the pre-gel solution remains in a macroscopic single phase (Tavares et al., 2005). However, further increases in the concentration of the soluble polysaccharides often result in weakening of the gel networks (van den Berg et al., 2007a). Spotti et al. (2012) and Fitzsimons et al. (2008) have shown that low concentrations of the non-gelling polysaccharide such as Espina Corona gum and guar gum, respectively, can substantially increase the gel strength of networks formed by thermal denaturation of whey protein isolate (WPI). Monteiro, Tavares, Evtuguin, Moreno, and Lopes da Silva (2005) found (by oscillatory rheology) that galactomannans of different molecular weights increase the solid character of WPI gels, this increase having a strong relationship with the nature and molecular weight of the galactomannan.

3.4. Mechanical parameters of conjugate gels

Since WPI/DX conjugate gels with different DX concentrations had similar behaviour for each reaction time studied, Fig. 3B shows only conjugate gels of 12% (w/w) WPI with 7.2% (w/w) DX added.

Since conjugate gels showed no fracture, the only mechanical parameter calculated from the uniaxial compression test was Young's modulus (because it is not calculated at the fracture point) (Fig. 4B). Both DX concentration and reaction time influenced this parameter. Young's modulus decreased with increasing DX concentration and reaction time. Many factors might promote the weakening of WPI gel network in conjugate gels. On the one hand, a loss of protein native structure could occur as a consequence of both days of incubation at 60 °C and the coupling of dextran. Because of this loss of native structure, the protein could take another shape, where sulfhydryl groups, which are primarily responsible for the gel network, are not fully available for covalent interactions. These results are similar to those obtained by Sun et al. (2011) by oscillatory rheology, where the value of storage modulus G′ (which measures solid behaviour) of WPI/DX conjugates was smaller than the G′ value obtained by WPI alone. According to this study, hydrophobic interactions, which play an important role in thermal aggregation and gelation of globular protein solutions, could be affected during Maillard reaction. Since a voluminous part of the polysaccharide is hydrophilic, the interactions between the hydrophobic amino acids are weakened, which could prevent the formation of aggregates between protein molecules. In our study, it

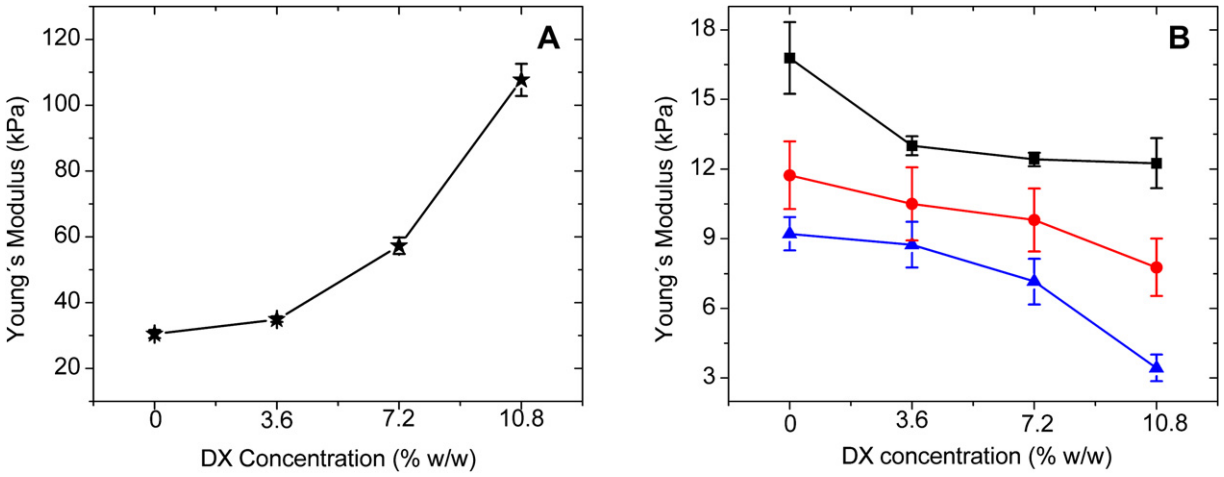

Fig. 4. Young's modulus vs dextran concentration for WPI/DX mixed gels (★) (A) and for WPI/DX conjugate gels from dry blends incubated for 2 days (■), 5 days (●) and 9 days (▲) (B).

Table 2
Mechanical properties of WPI/DX mixed gels at different DX (15–25 kDa) concentrations.

DX conc (% w/w)	σ_M (kPa)	ε_M	W_F
0	58.4 ± 6.5[a]	0.71 ± 0.01[a]	15.6 ± 1.4[a]
3.6	72.6 ± 7.6[a]	0.64 ± 0.02[a]	15.8 ± 1.5[a]
7.2	97.5 ± 3.6[b]	0.69 ± 0.03[a]	27.8 ± 1.9[b]
10.8	118.1 ± 12.1[c]	0.65 ± 0.01[a]	32.9 ± 3.8[c]

Conc = Concentration. Values with the same letter did not show differences with Duncan's test with $\alpha = 0.05$.

Table 3
Initial stress and relaxation modulus of WPI gels at 12% (w/w) with 7.2% (w/w) DX added.

Days	σ_{Hi} (kPa)	G (kPa)
0	2.86 ± 0.60[a]	57.2 ± 12.0[a]
2	0.67 ± 0.07[b]	13.4 ± 1.3[b]
5	0.50 ± 0.04[c]	10.0 ± 0.8[c]
9	0.31 ± 0.04[d]	6.1 ± 0.9[d]

The values with the same letter did not have differences with Duncan's test with $\alpha = 0.05$.

was found that the hydrophilic part of the studied DX did not weaken the gel network, since WPI/DX mixed gels had an increase in gel hardness with respect to WPI alone.

3.5. Stress relaxation of WPI/DX mixed and conjugate gels

In Fig. 5, examples of the curves obtained in stress relaxation test conducted in uniaxial compression for WPI/DX mixed and conjugate gels are shown. As in Figs. 3B and 5 only show curves for WPI/DX mixed and conjugate gels obtained with 12% w/w WPI and 7.2% w/w DX, since other DX concentrations (mixed and conjugate systems) had similar behaviour.

It can be observed that the shape of curves, i.e., the way in which gels relax their structure, is very similar for WPI/DX mixed (0 days) and conjugate gels (2, 5 and 9 days). WPI/DX mixed gel (0 days) had a higher initial stress as compared with WPI/DX conjugate gels (2, 5 and 9 days). Both initial stress (σ_{Hi}) and initial relaxation modulus (G_0) are shown in Table 3. Statistically significant differences were found between the different reaction times for these parameters. The initial stress of the conjugate gels decreased with increasing DX concentration and reaction time, that is to say, when the reaction had progressed further. These results are similar to those found in Young's modulus values (Fig. 4B), since mixed gels are stronger than conjugate gels, and the latter lost firmness as Maillard reaction progressed.

3.6. Confocal scanning laser microscopy images of mixed and conjugate gels

Confocal scanning laser microscopy images of 12% WPI gels showed a continuous uninterrupted image where the protein network can not be observed, probably due to the fact that

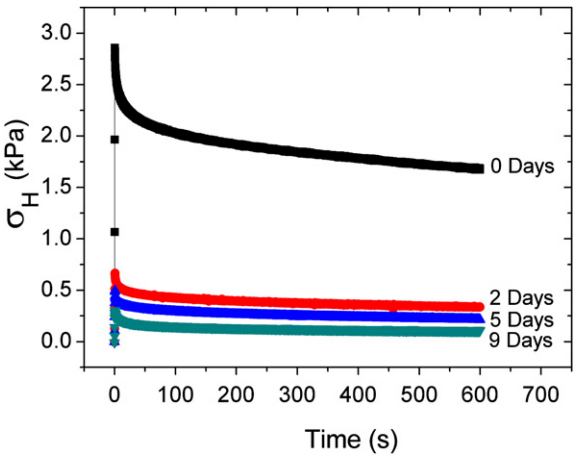

Fig. 5. Stress vs time curves of stress relaxation (applying 5% deformation) for mixed gel with 12% w/w WPI and 7.2% w/w DX (0 days) and conjugate gels with 12% w/w WPI and 7.2% w/w DX (2, 5 and 9 days).

filaments and pores making up the gel are smaller than the maximum resolution of the microscope (approximately 200 nm). Images of WPI/DX mixed and conjugate gels did not show changes in structure with respect to images of WPI alone gels (Figure not shown). WPI/DX mixed gel images did not reveal phase separation (at least, not visible with this microscopy) in the concentration range studied. In a previous investigation, heat mixed gels of WPI and Espina Corona gum (ECG), another neutral polysaccharide, added in much lower concentrations than the DX concentration range used in this work, showed phase separation when applying this method (Spotti et al., 2012). This phase separation was directly correlated with uniaxial compression test results since WPI/ECG mixed gels increased the hardness with increasing ECG concentration. In comparing both studies, as molecular weight of DX (15–25 kDa) is much smaller than the molecular weight of the ECG (1390 kDa), it can be inferred that this would be the reason why there is no phase separation in WPI/DX mixed gels. These results are similar to those found by Monteiro et al. (2005), who studied the effect of locust bean gum (a galactomannan) on the microstructure of heat-induced whey protein gels. In that study, the galactomannan with the lowest molecular mass (168 kDa) did not change the microstructural arrangement of the protein network. However, for the mixtures with galactomannan samples with higher molecular weights (1930–425 kDa), phase separation occurred with irregular masses of polysaccharide solution dispersed in a continuous protein matrix; the size of the area rich in polysaccharide increased as the molecular weight of the polysaccharide increased.

4. Conclusions

Maillard reaction was observed under all conditions studied. Reaction time greatly affected gelation properties. WPI/DX mixed gels fractured under uniaxial compression and mechanical properties at fracture were calculated, while conjugate gels did not fracture under the test conditions. In addition, Young's modulus values and stress-relaxation measurement allowed conjugate gels to be characterized and compared with mixed gels. It was observed that conjugate gels were less firm and hard. Confocal laser scanning microscopy did not show phase separation in the concentration range studied. Further studies where these structures could be analysed with other types of microscopes, such as scanning electron microscopy, remain to be conducted.

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