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ORIGINAL CONTRIBUTION

Gelation of whey protein concentrate in the presence of partially hydrolyzed waxy maize starch and urea at pH 7.5

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Abstract The effect of urea (50 and 100 g/l) and of partially hydrolyzed waxy maize starch (HWS, 100 and 150 g/l) on the heat-induced gelation of whey protein concentrate (WPC) at 100 g/l and pH 7.5 was investigated by small amplitude oscillatory measurements under heating to 80 °C and cooling to 25 °C, both treatments being followed by a stabilization period. Addition of urea contributed to the reduction of the values of the storage and loss moduli all over the heating/cooling treatment. On the contrary, addition of HWS alone led to higher moduli values. At the same HWS concentration, the increase of the moduli values was less pronounced when urea was present at 50 g/l. The microstructure of the systems was visualized by light microscopy at 25 °C, after the heating/cooling treatment. Addition of HWS affected the protein network in such a way that at 100 g/l, WPC/HWS mixed gels were visualized as a thick network with large pores, and at 150 g/l, the mixed gels were composed of denser strands with a larger number of smaller pores. However, when urea was also added to these mixed systems, homogeneous gels were imaged. Although leading to weaker gels, addition of urea seems to promote the compatibility of the two macromolecules.

Keywords Rheology · Light microscopy · Whey protein concentrate · Hydrolyzed waxy maize starch · Urea

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Introduction

Bovine whey, fluid produced from cheese manufacture, consists of a complex mixture of globular proteins, lactose, lipids, and minerals, in water. Whey can be processed into whey protein concentrates and isolates (protein contents higher than 90%) by simple drying or by removing lactose, lipids, and minerals. These concentrates (WPC) and isolates (WPI) have been widely used due to their functional properties, the most important of which is their gel-forming ability. Gelation of globular proteins has been extensively investigated and can be promoted by chemical, enzymatic, or thermal action [1].

Heat-induced gelation is the result of an irreversible aggregation process, which involves conformation changes, physical interactions, and eventually chemical reactions, and occurs at sufficient high protein concentrations. To form a macroscopic gel, the protein concentration should exceed a given critical value. However, to gain knowledge on the gelation phenomenon, many studies have focused on aggregation, which occurs at low nongelling concentrations. The aggregation process of β-lactoglobulin, the major whey protein that dominates the overall gelling behavior of whey proteins, has been studied at low concentration and shown to proceed in two steps [2–4]. At neutral pH and room temperature, β-lactoglobulin exists

primarily as a dimer [5, 6]. On heating, the dimers dissociate into monomers, which partially unfold. Changes in tertiary and secondary structures lead to the exposure of initially buried nonpolar groups on its surface [7–9], while the free sulfhydryl groups become reactive [8, 10, 11]. Small-angle neutron and X-ray scattering studies associated with light-scattering measurements indicated that the partially denatured β-lactoglobulin molecules are still globular in shape at the first step of the aggregation process and that variation in ionic strength (within 0.003–0.1 M NaCl concentrations), temperature (in the range of 70–90 °C), and protein concentration (0.4–30 g/l) had no effect on their size [2, 12].

In general, three types of intermolecular interactions control heat-induced aggregation and gelation of globular proteins. Intermolecular hydrophobic interactions arise when previously hidden nonpolar amino acid side chains are exposed as the macromolecular structure uncoils during thermal denaturation. This type of interaction was reported to be involved in the formation of primary aggregates between pH 6 and 9 [13]. Hydrogen bonds result from the interaction of polar amino acid side chains and are responsible for the network of gels formed at acid pHs [14]. Repulsive electrostatic interactions play an important role at pHs far from the isoelectric point of the protein and lead to the formation of the so-called fine-stranded network structures [15, 16]. Also, intermolecular covalent bonds result from reactions of sulfhydryl groups, which can form intermolecular disulfide linkages with other reactive sulfhydryl groups or through sulfhydryl–disulfide interchange reactions. These covalent cross-links, together with hydrophobic interactions, are the main determinants of the network of whey protein gels at neutral and alkaline pHs [14].

A three-stage mechanism was proposed to explain the formation of β-lactoglobulin homogeneous fine-stranded gels: initial protein unfolding, linear fibrillar aggregation, and random association of the fibrils. Due to the complexity observed for protein gelation, a fourth kinetic stage of de-mixing was suggested to occur as uniform networks give way to more heterogeneous morphologies [17].

Globular proteins can be denatured in highly concentrated solutions of certain low molecular weight substances such as urea and guanidine hydrochloride. Spontaneous gelation of WPI was observed in the presence of 6 M urea. Gels were formed more readily and the rigidity developed more rapidly as the pH was increased from 7 to 10 [18]. The relationship between protein stability and sulfhydryl group reactivity was examined for β-lactoglobulin in the presence of urea [19]. Extrinsic fluorescence intensity measurements in 0–8 M urea was used to estimate the free energy change for β-lactoglobulin unfolding in the absence of urea. Measurements of the rate of sulfhydryl/disulfide exchange in 0–8 M urea at pH 3 were used to estimate the free energy change for exposing sulfhydryl groups of β-lactoglobulin. Unfolding profiles detected by fluorescence measurements showed that the protein unfolds at lower urea concentrations as compared with those required to increase sulfhydryl group reactivity [19]. Protein unfolding in denaturing solvents such as urea was attributed to a better solvating ability of nonpolar groups by urea than water [20].

In the frame of a project aiming at developing environmentally friendly adhesives based on low-cost materials, a whey protein concentrate (WPC) and a partially hydrolyzed waxy maize starch (HWS) were selected to be studied in the present work. The effect of the addition of low concentrations of urea on the heat-induced gelation of the WPC sample and on the rheological and morphological properties of the final gels was investigated. The same study was performed on the more complex systems composed of WPC and HWS, with or without the addition of urea.

Mixtures of proteins and polysaccharides are often unstable and tend to de-mix into protein-rich and polysaccharide-rich phases with characteristic microstructure generally consisting of droplets of the solution of one of the components in a continuous “matrix” composed of the solution of the other [21, 22]. When in a mixed biopolymer solution one or both components are gel-forming polymers, de-mixing and gelation become competing processes and alternative gel microstructures are possible [23, 24]. Studies on the rheological properties [25] and the relationships between the microstructure and rheology [26, 27] of mixed systems of particulate β-lactoglobulin and nongelling potato amylopectins at pH 5.4 were reported. The rheological properties and the network connectivity of β-lactoglobulin were influenced both by the properties and concentration of the potato amylopectin. In the present work, WPC, WPC/urea, WPC/HWS, and WPC/HWS/urea systems were studied far from the isoelectric point of the proteins at pH 7.5. To the authors’ knowledge, the effect of urea on gelling systems of WPC and WPC/partially hydrolyzed nongelling starch has not been addressed before.

Materials and methods

Materials

A commercial sample of whey protein concentrate, Alacen™ 450, was supplied by Probiótica Produtos Naturais, São Paulo, Brazil. According to the manufacturer, the concentrate contains 82.3% protein, 3.4% lactose, 7.5% fat, and 2.8% ash. Waxy maize starch was given by Corn

Products Brazil (São Paulo, Brazil) and contains 2% amylose and 0.2% ash. Reagent grade urea was supplied by Vetec Química Fina (Rio de Janeiro, Brazil).

Hydrolysis of waxy maize starch

A waxy maize starch suspension was prepared at 7% (w/w) concentration, gelatinized at 90 °C for 90 min, and submitted to ultrasound radiation in a 750-W Cole Parmer Processor (Vernon Hills, IL, USA) at 25 °C for 15 min. The partially hydrolyzed waxy maize starch sample was recovered by precipitation in ethyl alcohol and dried in an oven at 50 °C for 24 h. The intrinsic viscosity, $[\eta]$, determined in 1 N KOH solution at 25 °C using Huggins' extrapolation, was 0.35±0.02 dl/g.

Preparation of samples

WPC suspensions (100 g/l in dry weight basis) were prepared in water by gently stirring the powder in distilled water at room temperature overnight. To prepare WPC/urea samples (100 g/l WPC and 50 or 100 g/l urea, dry weight basis), the necessary weight of urea was added as a powder to a previously prepared WPC suspension, and the resulting mixture was stirred for an additional period of 10 min. To prepare WPC/HWS (100 g/l WPC and 100 or 150 g/l HWS, dry weight basis), HWS solutions were prepared under stirring at 80 °C for 20 min. After cooling to room temperature, WPC was added as a powder to the HWS solution, and the resulting suspension was maintained under gentle stirring at room temperature overnight. For the preparation of WPC/urea/HWS mixtures (100 g/l WPC, 100 or 150 g/l HWS, 50 g/l urea, dry weight basis), the procedure was similar but the necessary weight of urea was added to the suspension. For all the preparations described above, the pH was adjusted to 7.5 with 1 M NaOH, and sodium azide (5 ppm) was added to avoid bacterial growth. The samples were degassed to remove air bubbles.

Rheological measurements

Rheological measurements were carried out in a Carri-Med stress-controlled rheometer CSL50 (Newcastle, UK) fitted with cone-and-plate geometry (cone angle of 2°, 4.0 cm in diameter, gap of 55 μm), within the linear viscoelastic range measured experimentally. WPC alone, WPC/urea, WPC/HWS, or WPC/urea/HWS suspensions were covered with a layer of paraffin oil to prevent water evaporation and submitted to a temperature ramp from 25 to 80 °C at a rate of 2 °C/min, at 6.28 rad/s. The temperature was maintained at 80 °C for 3.5 h, and then a frequency sweep was performed over the 0.06- to 62.83-rad/s range at 80 °C. The temperature was decreased to 25 °C at a rate of 2 °C/min. After an equilibration period of 1 h at 25 °C, a frequency sweep was recorded at this temperature over the 0.06- to 62.83-rad/s range. The strain amplitude was maintained at 1%. To check that the data recorded during the frequency sweeps ("mechanical spectra") were obtained within the linearity domain of the systems all over the frequency range, the first-order approximation of the mechanical equivalent of Kronig–Kramers relationship was used to calculate $G''(\omega)$ from G' value taken at the lowest frequency, and the calculated values were compared to the measured ones. The approximation is given by Eq. (1) [28].

$$G''(\omega) = \frac{\pi}{2}\frac{dG'(\omega)}{d\,ln\,(\omega)} \tag{1}$$

The experiments were carried out in duplicate with the results reported as the average of the measurements. The variation coefficient was about 10%.

Light microscopy

The samples analyzed by light microscopy were prepared similarly as described in "Preparation of samples." Rhodamine B (~2 mg) was added to WPC suspensions before stirring overnight. This procedure was followed for the other mixtures. After degassing, drops were poured on a slide, covered with a glass cover slip, and hermetically sealed to prevent evaporation. After a heat treatment similar to that used for the rheological measurements, the samples were allowed to stand for 30 min before examination in a Carl Zeiss, Axioplan 2 microscope (Göttingen, Germany) by light microscopy under direct fluorescence.

Results and discussion

Before preparing the HWS sample, gelatinized waxy maize starch was submitted to ultrasound radiation over a 30-min period, during which aliquots were taken from the reaction batch and had their viscosities measured. The viscosity of the sample obtained after the 15-min reaction time was considered low enough to achieve the desired flow properties. Moreover, when the HWS sample at 300 g/l concentration was submitted to heating and cooling treatments in the rheometer (data not shown), the loss modulus $G''(\omega)$ values were higher than the storage modulus $G'(\omega)$ values, over the 0.20- to 62.83-rad/s range. This behavior revealed the nongelling liquid-like character of the sample even at a high concentration.

As observed for other systems [29], during the ascending temperature ramp step for WPC alone or in admixture with urea (50 and 100 g/l), the moduli remain very small up to approximately 80 °C. Figure 1a,b shows the time evolution of G' and G'' at 80 °C for those systems. In the beginning

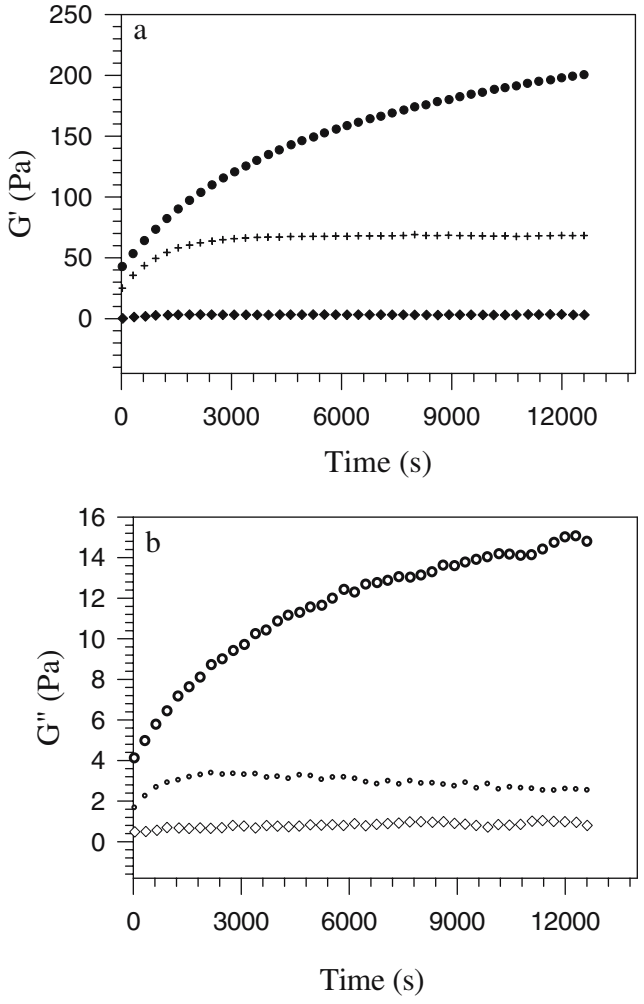

Fig. 1 Variation of **a** G′ and **b** G″ as a function of time for WPC (*shaded circle*, *open circle*) and WPC/urea systems with urea at 50 g/l (*plus signs*, *dots*) and at 100 g/l (*shaded diamond*, *open diamond*) concentrations at 80 °C, 6.28 rad/s, and 1% strain amplitude

of this step, G' is higher than G'', which indicates that gelation has already occurred during the heating period and that the subsequent evolution of G' is related to the strengthening of the three-dimensional network. At the end of this cure step, G' has not reached a plateau for WPC alone. The addition of urea caused a decrease of both moduli, which depended on urea concentration; the higher this concentration, the lower were the moduli values. But, contrary to the WPC system, when urea was present, G' reached a plateau.

As explained in the "Introduction", covalent and non-covalent interactions are involved in heat-denaturing and aggregation of globular proteins. Structural changes can be induced from the exposure of the protein to pH ≥6.7 [30], denaturants [8], high temperatures [8, 20], and high pressures [31]. Under the conditions used in the present experiments (pH 7.5, temperature of 80 °C, and urea at 0.8 and 1.6 M concentrations), denaturation and formation of disulfide linkages should be favored, as it is known that urea interferes with hydrogen/hydrophobic bonding [18]. In a study published recently [32], WPC gels formed by disulfide bonding only developed slowly during heating and were softer than WPC control gels, which seems to be in accordance with the present results.

Addition of HWS to WPC brought on some modifications to the gelation behavior, and this effect depended on the WPC/HWS ratio. It should be pointed out here that HWS is a nongelling starch sample; therefore, it is the gelation of WPC that is being monitored. As observed with WPC alone, for these mixed systems gelation occurred during the heating step, but the values of the moduli were considerably higher in this case, when the temperature reached 80 °C. Also, the curing experiments revealed that the gels were still evolving after 3.5 h at 80 °C (Fig. 2). At the end of the curing process, for the WPC/100 g/l HWS system, G' reached a value 1.6 times higher than that

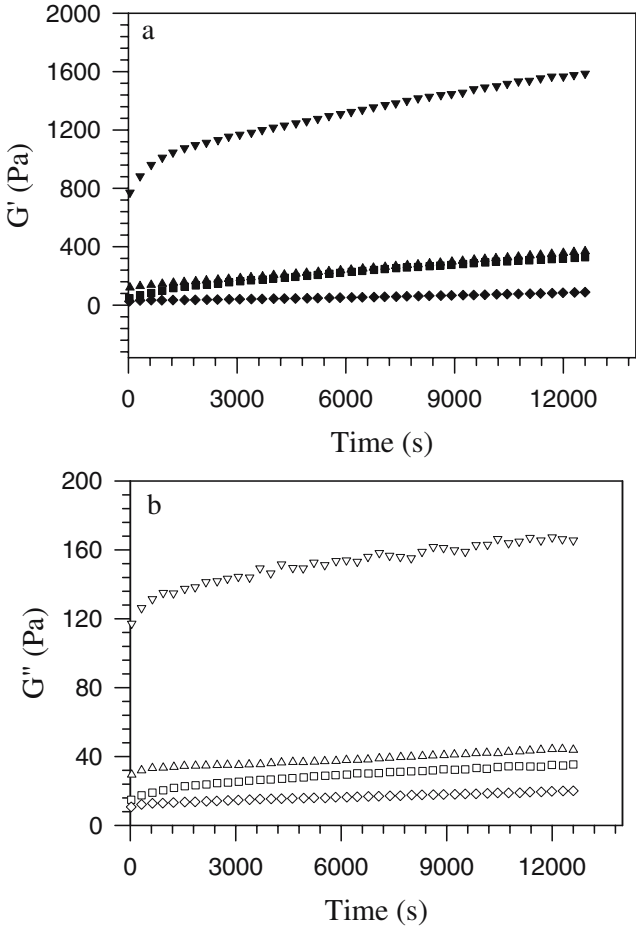

Fig. 2 Variation of **a** G' and **b** G'' as a function of time for WPC/HWS systems with HWS at 100 g/l (*shaded square*, *open square*) and 150 g/l (*shaded inverted triangle*, *open inverted triangle*) concentrations, and WPC/HWS/urea systems with urea at 50 g/l and HWS at 100 g/l (*shaded diamond*, *open diamond*) and 150 g/l (*shaded triangle*, *open triangle*) concentrations at 80 °C, 6.28 rad/s, and 1% strain amplitude

obtained for WPC system, whereas in the case of the WPC/150 g/l HWS system, G' was 7.9 times higher. When urea (50 g/l) was added to the mixtures, gel evolution was slowed down and, as was the case for WPC alone, a decrease in the moduli values was observed (Fig. 2).

Quenching the gels to 25 °C caused an enhancement of the moduli pointing to the importance of hydrogen bonds in gel strengthening (Fig. 3). However, the increase of the moduli was not the same for all gels; the higher the concentration of HWS in the mixed gel, the steeper was this increase. Also, for similar HWS concentrations, the increase was less pronounced when urea at 50 g/l was present. This may indicate that urea interferes with hydrogen bond formation, as pointed above, or that for the networks formed without stabilizing hydrophobic interactions, hydrogen bond formation was more difficult.

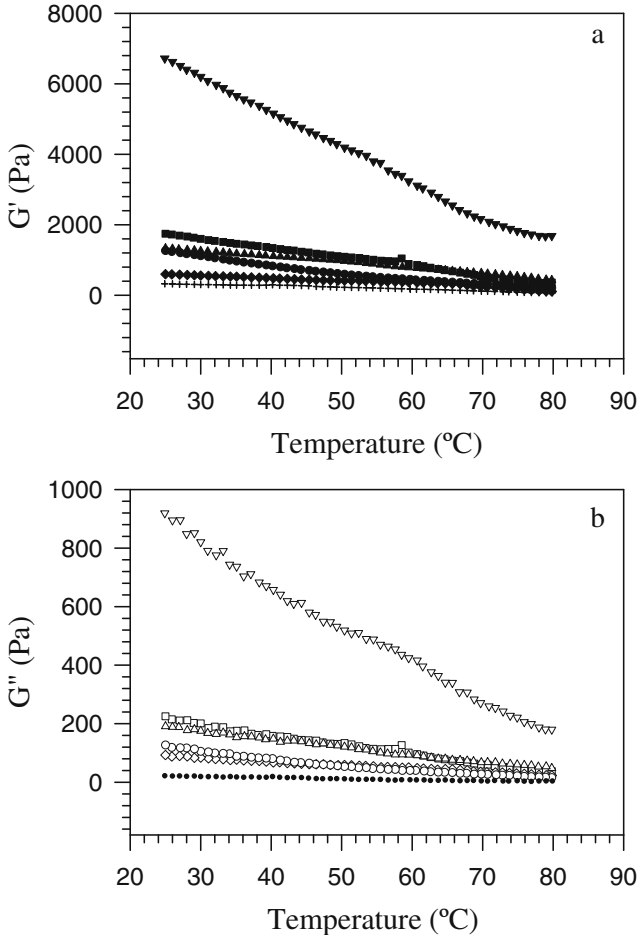

Fig. 3 Variation of **a** G' and **b** G'' as the temperature is decreased from 80 to 25 °C for WPC (*shaded circle*, *open circle*), WPC/urea with urea at 50 g/l concentration (*plus signs*, *dots*), WPC/HWS systems with HWS at 100 g/l (*shaded square*, *open square*) and 150 g/l (*shaded inverted triangle*, *open inverted triangle*), and WPC// HWS/urea systems with urea at 50 g/l and HWS at 100 g/l (*shaded diamond*, *open diamond*) and 150 g/l (*shaded triangle*, *open triangle*) concentrations at 6.28 rad/s and 1% strain amplitude

After an equilibration period of 1 h, no significant evolution of the moduli was observed (data not shown), which allowed the mechanical spectra to be recorded over the 0.06- to 62.83-rad/s frequency range. For all the mixed systems studied, the mechanical spectra showed a section of the viscoelastic plateau, with $G'>G''$ (Figs. 4 and 5). The general effect of the addition of HWS was an increase in gel rigidity (as seen by the increase of G') and a decrease of its elastic character. In Fig. 6, *tan* δ increases slightly at low frequencies and even more markedly at higher frequencies. Again, when urea was present, the moduli were lowered (compare data in Figs. 4 and 5).

The shapes of the storage moduli, $G'(\omega)$, were similar for all systems, while the shapes of the loss moduli function, $G''(\omega)$, differed. At low frequencies, $G''(\omega)$ had an almost constant value but, as frequency increased, the values of the moduli also increased. This increase was not very pronounced for WPC (Fig. 4) or WPC/urea at 50 g/l (Fig. 5); however, for WPC/HWS gels, the values of $G''(\omega)$ increased more rapidly and sooner (at lower frequencies) when the HWS concentration was lower (Fig. 4). This effect was more pronounced in mixed systems with urea (Fig. 5).

In Fig. 6, it may be observed that, for WPC/urea gel (Fig. 6b), *tan* δ values decreased in relation to WPC alone (Fig. 6a), over the frequency window studied. For both WPC/HWS mixed gels with urea (Fig. 6b), *tan* δ decreased slightly at low frequencies, but increased steeply from 1 rad/s. This increase was still more pronounced for the gel with the lower HWS concentration, which indicates its higher viscous character.

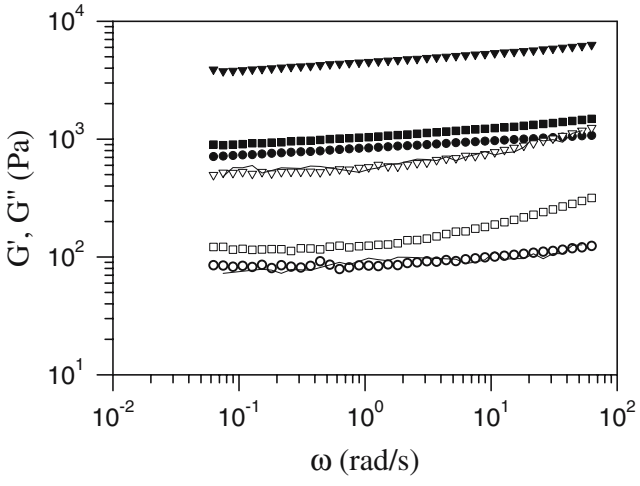

Fig. 4 Mechanical spectra of the gelled systems at 25 °C (1% strain amplitude) for WPC (*shaded circle*, *open circle*) and WPC/HWS system with HWS at 100 g/l (*shaded square*, *open square*) and at 150 g/l (*shaded inverted triangle*, *open inverted triangle*) concentrations. The *lines* represent $G''(\omega)$ calculated from $G'(\omega)$ data in the case of WPC, and WPC/HWS system with HWS at 150 g/l, using Eq. (1). The results are not shown for the other system for the sake of clarity

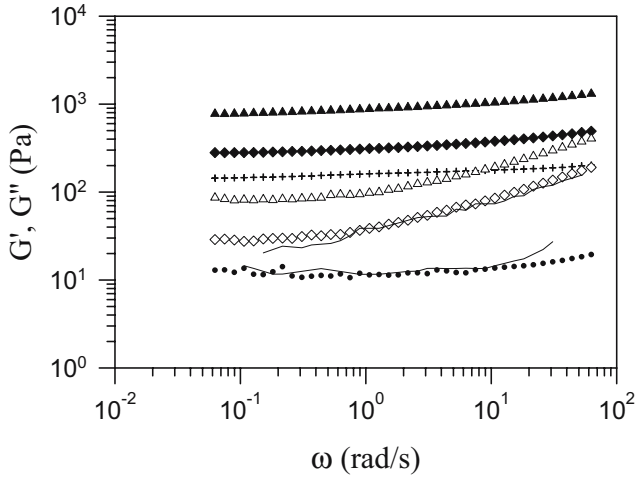

Fig. 5 Mechanical spectra of the gelled systems at 25 °C (1% strain amplitude) for WPC/urea (*plus signs*, *dots*) and WPC/HWS/urea systems with urea at 50 g/l concentration and HWS at 100 g/l (*shaded diamond*, *open diamond*), and at 150 g/l (*shaded triangle*, *open triangle*) concentrations. The *lines* represent $G''(\omega)$ calculated from $G'(\omega)$ data in the case of WPC/urea and WPC/HWS/urea system with HWS at 100 g/l using Eq. (1). The results are not shown for the other system for the sake of clarity

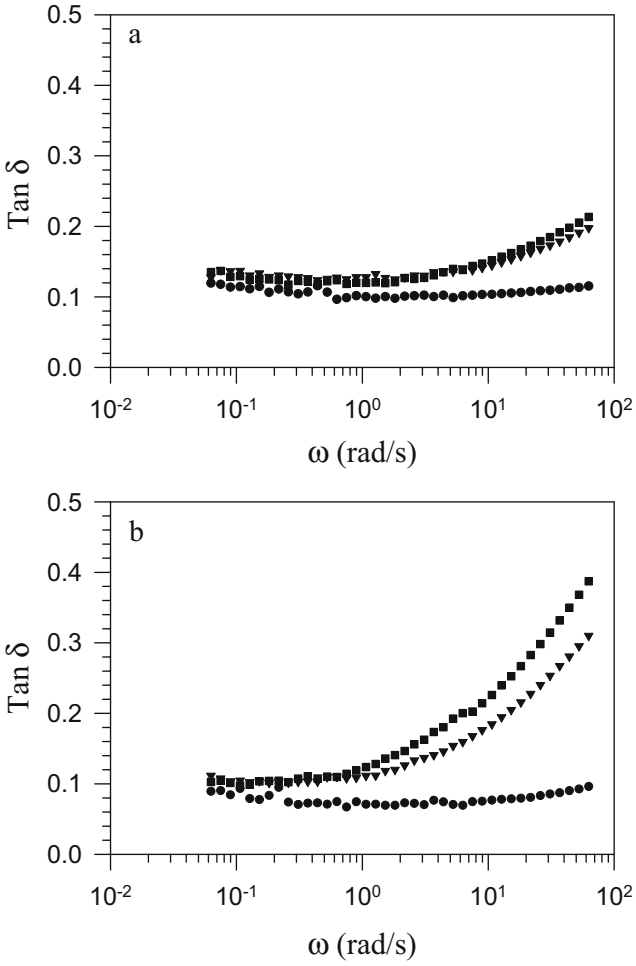

Fig. 6 Mechanical spectra (tan δ) of the gelled systems at 25 °C (1% strain amplitude) for WPC and WPC/HWS systems (**a**) and WPC/urea and WPC/HWS/urea systems, with urea at 50 g/l concentration (**b**). HWS at 0 g/l (*shaded circle*), 100 g/l (*shaded square*), and at 150 g/l (*shaded inverted triangle*) concentrations

Linearity of the viscoelastic behavior was checked. As shown in Figs. 4 and 5, a good agreement was obtained between experimental $G''(\omega)$ values and values of $G''(\omega)$ calculated from G' at the lowest frequency using Eq. 1. This means that the spectra were recorded below the linearity limit and that no significant structural changes occurred in the gels during this record. As shown in Table 1, the height of the viscoelastic plateau $\left(G_N^0\right)$ at 25 °C increased with HWS concentration in the systems, without urea (compare the values obtained for WPC, WPC/HWS at 100 g/l and WPC/HWS at 150 g/l), and with urea at 50 g/l (compare the values obtained for WPC/urea, WPC/HWS at 100 g/l/urea, and WPC/HWS at 150 g/l/urea). However, the values of G_N^0 were higher for systems without urea.

The gelled systems were observed by light microscopy under direct fluorescence (200× objective). Light and dark regions correspond to protein-rich and protein-depleted phases, respectively. In Fig. 7a, gelled WPC at 100 g/l forms a coarse, homogeneous network. According to some authors [33], non-covalent interactions become of increasing importance for treatments at temperatures higher than 75 °C at neutral pH. When urea (50 g/l) was added, a finer network was visualized (Fig. 7b). This finer structure should be expected because, in this case, linkages between the protein molecules are more limited due to the interference of urea on non-covalent bonding; it appears that the more the disulfide bonding dominates in WPC gels, the more fine-stranded the gels are [32].

For mixed gels of WPC/HWS, the micrographs show that the HWS influences the state of aggregation of WPC proteins; the effect depends on HWS concentration in the system. For the systems with no urea added, a phase separation was observed with disperse HWS-enriched regions (dark regions) surrounded by a thick, continuous matrix, composed of WPC-rich gel (light regions). These

Table 1 Rheological parameters extracted from the analysis of the mechanical spectra of the gels recorded at 25 °C, after the heating/cooling treatment

Sample[a]	$G_N^0 = 1/J_N^0(Pa)$[b]
WPC	711.4
WPC/HWS at 100 g/l	899.2
WPC/HWS at 150 g/l	3,895.0
WPC/urea	144.4
WPC/HWS at 100 g l^{-1}/urea	280.7
WPC/HWS at 150 g l^{-1}/urea	775.2

[a] WPC containing 100 g/l whey protein concentrate; urea added at 50 g/l concentration

[b] taken as the value of G' at the lowest frequency

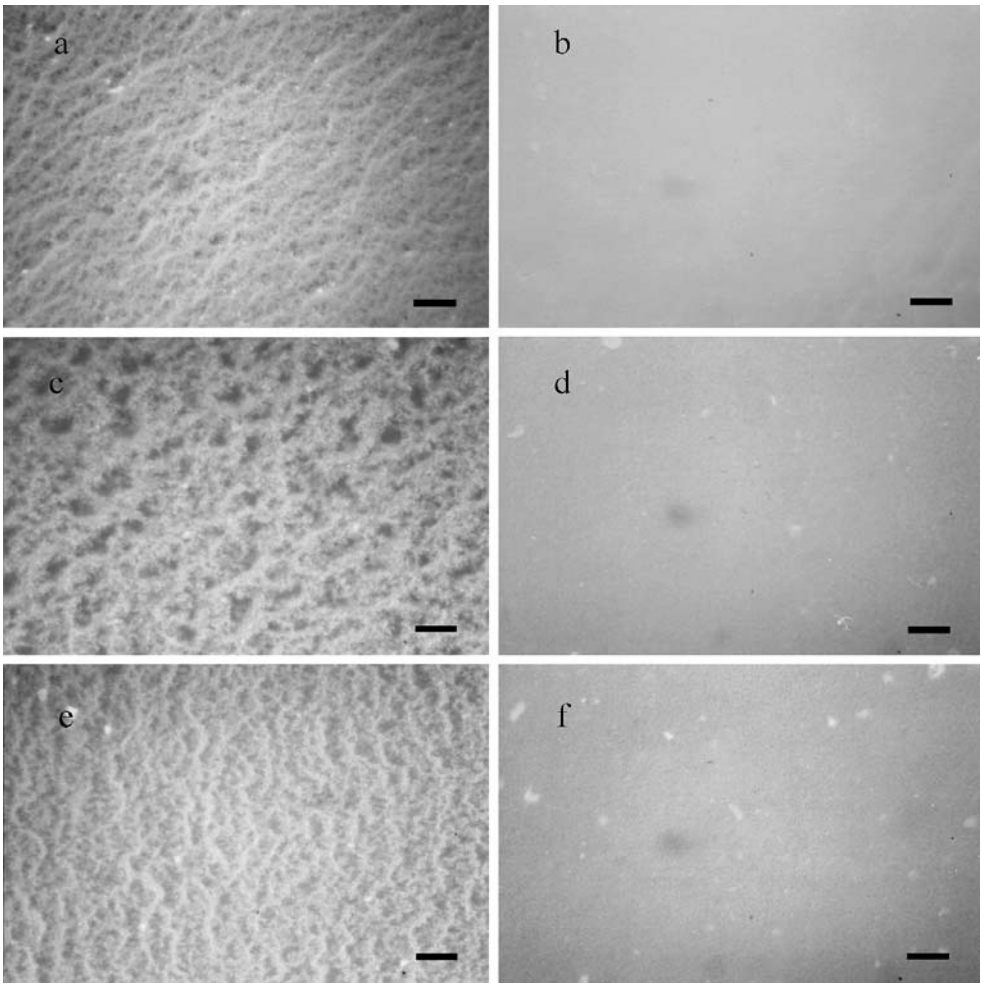

Fig. 7 Light micrographs under direct fluorescence of WPC at 100 g/l after the heating/cooling treatment; alone (**a**), with urea at 50 g/l (**b**), with HWS at 100 g/l (**c**), with HWS at 100 g/l and urea at 50 g/l (**d**), with HWS at 150 g/l (**e**), with HWS at 150 g/l and urea at 50 g/l (**f**)

gels are more heterogeneous than simple WPC gels. At the magnification used for the observations, it seems that for the lowest HWS concentration in the system (Fig. 7c), the structure presents thicker strands of aggregates and larger pores than for higher HWS concentration (Fig. 7e). With increasing polysaccharide concentration, a higher incompatibility with the proteins would be expected, since the polysaccharide-rich phase was increased. However, the opposite seems to occur; the microstructure of the mixed gel with HWS at 150 g/l appears as a phase-separated system with the dispersed HWS-rich phase of smaller dimensions suspended in a connected protein-rich network (Fig. 7e). One might suppose that, at some intermediate concentration of HWS (between 100 and 150 g/l), there could exist a critical concentration in which pores of maximum size would be formed. The smaller dimensions of the dispersed HWS-rich phase in the WPC/HWS at 150 g/l mixed gel are supported by the higher G' values observed for this system (Fig. 4), since a larger number of smaller inclusions would promote a protein network with higher connectivity.

The microstructures changed completely when urea was added (Fig. 7d,f). At the magnification used in the experiments, no phase separation could be observed, which seems to indicate that urea promotes a higher compatibility of WPC and HWS. Further experiments on the microstructure of these gels, at other length scales, would give useful information to a better understanding of these systems.

Conclusions

The influence of urea and partially hydrolyzed nongelling waxy maize starch on the heat-induced gelation and cooling of whey protein concentrate at 100 g/l concentration at pH 7.5 was investigated by small deformation oscillatory rheometry. Addition of urea at low concentrations resulted in weaker gels, whereas addition of HWS led to more rigid gels. In the latter case, gel connectivity, as reflected by the viscoelastic plateau moduli $\left(G_N^0\right)$ at 25 °C, varied by a factor of approximately 5 between WPC gel and the mixed WPC/HWS gel with the highest concentration of HWS. On the other hand, $tan\,\delta$ values indicated the higher viscous character of the mixed gels in relation to WPC gel alone. According to the light microscopy images, the main change in microstructure was observed at the lowest HWS

concentration for mixed gels without urea. This change might reflect the phase separation process occurring in the system before gelation. Although the rheological behavior was not qualitatively modified, at the scale of light microscopy, further increase in HWS concentration led to a denser protein network with the dispersed HWS-rich phase of smaller dimensions. In the presence of urea, the viscoelastic plateau moduli for WPC and WPC/HWS mixed gels were 3.3–5 times lower, which revealed weaker gel connectivities. The microstructures were completely changed when urea was added and resulted in homogeneous networks at the scale of light microscopy observation.

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