Combined effect of heat treatment and ionic strength on the functionality of whey proteins

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ABSTRACT

A 5% (wt/vol) whey protein isolate (WPI) dispersion (pH 6.5) with different concentrations of NaCl was submitted to dynamic heat treatment. Protein dispersions were characterized as to their rheological properties, particle sizes, morphology, denaturation temperatures, and protein surface hydrophobicity. At low ionic strength (<200 mmol/kg), gel elastic modulus increased and strongest gel stiffness was achieved. High salt concentrations lead to a weaker gel, whereas no gels at all were formed without salt. The gelation temperature was also influenced by ionic strength and an increase in denaturation temperature and thermal stability was also observed by using differential scanning calorimetry. Additionally, heat-induced changes in secondary structures upon salt augmentation were followed by Fourier transform infrared spectroscopy. Secondary structural elements estimations obtained from amide I assignments were correlated with those from amide III assignments. Upon salt increase, no differences in secondary structure were observed without heating, whereas upon heating and without salt increase, the Fourier transform infrared spectroscopy data revealed an increase in intermolecular β -sheets at the cost of β -turns and random coils, with no change in α -helical structures. However, NaCl addition along with dynamic heat treatment of WPI dispersion showed a stabilizing effect on the secondary structural elements of both amide I and amide III bands. Whey protein isolate dispersions in water were also characterized by transmission electron microscopy by a spherical shape with 2 populations (6 and 70 nm). Salt increase alone resulted in the formation of denser aggregates, whereas a transition from spherical/compact protein aggregates to linear ones was observed due to combined salt/heat

effect. The important size of these edifices was confirmed by microscopy and light-scattering techniques. Moreover, protein surface hydrophobicity related to the number of hydrophobic sites available decreased significantly. Finally, experimental results demonstrated the strong interaction between ionic strength and dynamic thermal treatment on protein functional properties and their careful adjustment could enable the food industry to effectively use WPI as a gelling agent.

Key words: whey protein, ionic strength, conformational change, temperature

INTRODUCTION

Whey proteins are among the most used and versatile sources of functional and nutritional food proteins. These represent a high nutritional profile with specific physiological properties and are widely used in the food industry (Madureira et al., 2010). Most probable food applications are in bakery products, processed meats, pasta, ice cream, confectionary, infant foods, spreads, dips, and beverages (Mulvihill and Ennis, 2003). Whey proteins are composed of 2 major fractions, β -LG and α -LA, representing, respectively, 54 and 21%. β -Lactoglobulin and α -LA are globular proteins consisting of 162 and 123 AA residues, respectively. With the advent of industrial filtration and separation methods, recovery and fractionation of whey proteins in their native form had become possible. Consequently, a wide variety of whey protein isolates (**WPI**), fractions, and hydrolysates are currently available (Nicorescu et al., 2008; Madureira et al., 2010).

Sodium chloride is an alimentary salt widely used in the dairy industry, as it is a major determinant of water activity. It is well known that microenvironments such as pH or salt, or both, can significantly alter the behavior and overall performance of proteins. In addition, whey proteins have an important cation-binding capacity (Hussain et al., 2011b). Addition of NaCl to whey proteins was found to generally enhance the formation of denser aggregates with turbidity, molecular

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size increase, and a protein solubility decrease (Vardhanabhuti and Foegeding, 2008; Hussain et al., 2011b). Sodium chloride could also affect the hydration process of proteins by electrostatic shielding and ion-specific hydrophobic interactions (Boye et al., 1996; Hussain et al., 2011b). Whey proteins in aqueous solution denature when heated, leading to an irreversible aggregation process and eventually a gel is formed above a critical protein concentration (Schmitt et al., 2007).

The aggregation process and gel structure are linked to various parameters (pH, protein concentration, temperature, and amount and type of salt, among others; Ikeda et al., 1999; Schmitt et al., 2011). Nevertheless, the mechanism of salt/heat-induced gelation of commercial whey proteins has been the subject of many studies in recent years. The denaturation/aggregation behavior of whey proteins were examined as a function of heating temperature in the presence of different salt media. In recent studies conducted specifically on whey proteins, an effort was made to understand the phenomenon and kinetics of salt/heat-induced gelation and its interpretation regarding protein functionality (Unterhaslberger et al., 2006; Schmitt et al., 2007; Nicorescu et al., 2008; Schmitt et al., 2011). Notwithstanding, the complete mechanism of salt/heat-induced gelation of whey proteins is still far from being understood. To better understand this process regarding rheological, morphological, and structural alterations in WPI, techniques able to probe their molecular structure and interactions are required. In earlier investigations, limited techniques have been used to determine the combined effect of heat and salt on the functionality of whey proteins. For example, rheological techniques have been used to determine gel properties such as gelation temperature and the storage (G') or loss modulus (G'') of salt/heat-induced WPI gels (Lorenzen and Schrader, 2006). Respectively, some researchers used various microscopic, calorimetric, and light-scattering techniques to characterize denaturation/aggregation behavior of whey proteins in an aqueous or salt environment, or both (Schmitt et al., 2007; Nicorescu et al., 2008).

Fluorescence titration using hydrophobicity probes such as 1-anilino-8-naphthalenesulfonate (**ANS**) has been proposed with success for the evaluation of whey protein surface hydrophobicity in variable media (Van Audenhaege et al., 2010; Schmitt et al., 2011). Globular proteins present compact and ordered conformations because of the presence of disulfide bridges, which give them a well-defined secondary and tertiary structure. The circular dichroism spectrum of proteins can reveal important characteristics of their secondary structure (Qi and Onwulata, 2011) but this technique presents some restrictions. Nonperiodic β -turn structures cannot

be distinguished by this methodology (Kavanagh et al., 2000). Fourier transform infrared (**FTIR**) spectroscopy is becoming an increasingly important method to study protein secondary structure, mostly by the analysis of the amide I region. With the use of FTIR, various elements of the secondary structures of whey proteins for amide I (Byler and Susi, 1986) and amide III (Cai and Singh, 1999, 2004) have already been determined.

In this paper, we present an investigation of the behavior leading up to heat/salt-induced gelation of WPI (5% wt/vol) as a function of variable NaCl concentrations (0 to 3%) and temperatures (30 to 90°C). The emphasis of this study is to relate the rheological and morphological properties of salt/heat-induced gels of WPI to the particle size, hydrophobicity, and interactions of the protein molecules in the gel network. For this purpose, a multilevel characterization was carried out by using FTIR spectroscopy, transmission electron microscopy (**TEM**), light-scattering techniques [dynamic light scattering (**DLS**) and static light scattering (**SLS**)], differential scanning calorimetry (**DSC**), and several other complementary techniques.

MATERIALS AND METHODS

Materials

Whey protein isolate powder was provided by Lactalis (Laval, France). The composition of the powder was (% by weight, wt/wt) 89.9% proteins (Kjeldahl N $\times$ 6.38), 0.5% fat, 1.0% lactose, 3.1% ash, and 5.5% moisture. Sodium chloride was furnished by VWR (Prolabo, Leuven, Belgium). Sodium azide (Merck KGaA, Darmstadt, Germany) was added as antimicrobial agent in each rehydration media (0.2 g/L). The pH (mean $\pm$ SD) of all systems studied was stable around 6.5 ± 2 .

Analyses were performed on totally rehydrated WPI powders at 5% wt (protein basis) in NaCl media at variable salt concentrations ranging between 0 and 3% salt. To obtain total rehydration of the powder, the dispersion was left under stirring overnight (12 h; Galiani et al., 2007) and referred to throughout this paper as WPI dispersion. To investigate the effect of heat treatment on WPI dispersions and particularly on the formation of heat-induced aggregates by TEM, DLS, and protein surface hydrophobicity (**PSH**), supernatants were collected (i.e., the soluble fraction). Whey protein isolate dispersions were transferred into 22-mL glass vials (with a screw top and solid cap) and heated in a water bath with a thermometer without stirring up to 90°C. Afterwards, the samples were immediately cooled in ice water to 4°C and measurements for the

characterization of dispersion properties were immediately carried out.

Particle Size and Structure Determination

Particle Size. Particle sizes of WPI dispersions were measured at 30 and 90°C using 2 different pieces of light-scattering equipment. For the smallest sizes (between 0.5 and 400 nm), a Nanosizer ZS particle size analyzer (HPPS 5001; Malvern Instruments Ltd., Malvern, UK) was used. Intensity autocorrelation functions were analyzed by a CONTIN algorithm to determine the distribution of translational z-averaged diffusion coefficient of particles (D_T). From the algorithm, the determination of the hydrodynamic diameter (D_h) was deduced from the hydrodynamic radius (R_h), $D_h = 2 \times R_h$. For the largest sizes (between 0.1 and 1,000 μm), a laser diffraction instrument (Mastersizer S; Malvern Instruments Ltd.) with a 5-mW He-Ne laser operating at a wavelength of 632.8 nm and 300RF lens was used. Almost 50 mL of WPI dispersion was diluted in 200 mL to obtain a correct obscuration (around 15).

Transmission Electron Microscopy. Microstructures of WPI dispersions under NaCl environments were determined at various temperatures (30 to 90°C) by TEM (CM20 Philips; 25,000 $\times$ magnification; Koninklijke Philips Electronics NV, Amsterdam, the Netherlands) using a negative staining method. One drop of dispersion was deposited onto a carbon-coated copper grid (200 mesh, 3-mm diameter). The excess of product was removed after 30 s using filter paper. Subsequently, 1 drop of uranyl acetate (2%) was placed on the grid for 2 min. After drying the grid at room temperature for 5 min, micrographs were made using TEM operating at 200 kV. Images were recorded using an Olympus TEM CCD camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany). Image contrast was improved by contrast stretching with Adobe Photoshop Elements 2.0 software (Adobe Systems Inc., San Jose, CA).

Rheological Measurements

Rheological measurements were performed with a Kinexus rotational rheometer (KNX 2100 UK; Malvern Instruments Ltd.). The rheometer was equipped with a temperature control unit (Peltier effect), allowing the obtaining of very precise temperature ramp. The time-temperature course of gel formation was characterized by small-deformation oscillatory measurements (0.01% strain; 1.0 Hz) of the G' using parallel plate geometry CP2/50 (cone and plate geometry with a 50-mm diameter and a cone angle of 2°). The gelation temperature was defined as the temperature at which the G' starts

to increase. Various salt dispersions (from 0 to 3%) were heated from 30 to 90°C at a rate of 1°C/min. Paraffin oil was applied to the sample exposed surface to prevent water evaporation (Michalski et al., 2002).

Thermal Denaturation

Thermograms of WPI dispersions in presence or absence of salt were studied by DSC using a micro DSC III (Setaram Instrumentation, Caluire, France). Whey protein isolate dispersions (850 mg) were hermetically sealed in a pan (Hastelloy C276; volume = 1 cm^3), and the reference pan was filled with water (Milli-Q water; Millipore Corp., Billerica, MA) or appropriate salt solutions, or both. After equilibration for 10 min at 10°C, the temperature was raised to 90°C at a heating rate of 1.2°C/min and then cooled to 30°C at 2°C/min. The total calorimetric apparent enthalpy change (ΔH) was obtained by integrating the area of the thermogram using a straight line between the onset and the final temperature of the thermal transition. Also, the denaturation temperatures (T_D) were determined from the integration of the calorimetric thermograms.

Surface Hydrophobicity Index Determination

For PSH determination, diluted WPI dispersions (0.05%) in quartz cells were placed in a spectrofluorometer (F2500; Hitachi Ltd., Tokyo, Japan) and aliquots of ANS were successively added (from 0 to 50 $\mu\text{mol/L}$; Schmitt et al., 2009). Titration with ANS was monitored at an excitation wavelength (λ_{ex}) of 390 nm and emission wavelength (λ_{em}) of 480 nm. The relative fluorescence intensity reading was adjusted to 1 when 50 μL of ANS solution was added to PBS (at 0.10 mM and pH 7). Analysis of binding data was performed to obtain the maximum fluorescence attainable at saturating ANS concentration (F_{max}). The F_{max} is a function of the number of hydrophobic sites accessible to the spectroscopic marker. The ANS concentration required to obtain half the value of F_{max} is considered to be the apparent dissociation constant of the fluorescent ANS-protein complex (K_d). The ratio F_{max}/K_d or PSH represents a cumulative index of surface hydrophobicity of protein, taking into account both the number and affinity of hydrophobic sites.

Protein Secondary Structure

Equipment. Protein secondary structures were followed by FTIR spectroscopy. Scans were obtained with a Tensor 27 mid-FTIR Bruker spectrometer (Bruker AXS GmbH, Karlsruhe, Germany) equipped with an

attenuated total reflectance (ATR) cell and a mercury cadmium telluride (MCT) detector cooled with liquid N₂. The scanning rate was 20 kHz and 126 scans were used for reference and samples between 4,000 and 850 cm⁻¹. The nominal instrument resolution was 2 cm⁻¹. A rheostat (Haake DC 30; Meyer-Haake GmbH Medical Innovations, Ober-Mörlen, Germany) was connected with the ATR cell to regulate the temperature between 30 and 90°C at the rate of 2°C rise per measure (O-PRO Protein Dynamics; Bruker AXS GmbH). First, references were recorded for water or NaCl solutions according to their concentrations. Then, 20 µL of WPI dispersion was put on the ZnSe crystal of the optical cell. The same temperature ramp was performed for references and samples. Three to 5 separate experiments were done for each salt concentration.

Data Treatment. All treatments were carried out using OPUS software (Bruker AXS GmbH). Raw absorbance spectra were smoothed using a 9-point Savitzky-Golay smoothing function. Spectra were cut between 1,720 and 1,580 cm⁻¹ or between 1,190 and 1,390 cm⁻¹ for analysis of amide I and amide III bands, respectively. Elastic baseline correction using 200 points was then applied to spectra. After that, spectra were centered and normalized using OPUS software. Second-derivative spectra were calculated on centered and normalized data to identify individual peak positions. Finally, the treated spectra were deconvoluted by a nonlinear regression curve-fitting program of Gaussian peaks to the original spectra. Integrated areas of each peak were calculated and related to secondary structural features (Byler and Susi, 1986; Cai and Singh, 1999; Hussain et al., 2011a).

Statistical Analyses

Statistical analysis was carried out by using the software KyPlot version 2.0 (<http://www.kyenslab.com/en/products/kyplot.html>). For comparisons between dispersion in water and other NaCl concentration, parametric multiple tests were performed (Dunnett test with whey dispersions in water as control). The level of statistical significance was $P < 0.05$.

RESULTS

Particle Sizes of WPI Dispersions

Particle sizes were determined by DLS or SLS, or both, to measure the D_h or particle diameters, or both (Table 1). The choice of the technique (DLS or SLS) depended mainly on the particle size range. Aggregates larger than 500 nm could not be correctly measured by

DLS. Conversely, aggregates under 200 nm were not correctly evaluated by SLS. The distributions obtained in water at 30°C were bimodal. Indeed, 2 populations were systematically observed. The first peak at 6 nm was significantly less represented than the second peak at 69 nm. Similar size was also observed by the analysis of a significant number of particles from TEM images (≈ 70 nm). For 0.50% salt (86 mmol/kg), similar D_h were measured. Nevertheless, the second peak (72 nm) was more important and represented more than 90% of the total particles. Concurrently, the major second peak exhibited a D_h of 160 and 316 nm for 1% (172 mmol/kg) and 1.5% salt (258 mmol/kg), respectively. As a consequence, the DLS technique was still possible up to 1.5% salt (258 mmol/kg) but not for higher concentrations, where SLS was more appropriate. For higher salt concentrations, similar particle sizes presenting 2 populations were found by SLS. The first population was related to small aggregates (420 nm) already noticed by DLS for lower salt concentrations. The second and most important population was related to large aggregates (around 35 µm) impossible to detect by DLS. These statements are reinforced with the images of TEM.

Particle sizes of heated WPI dispersions were also measured. Distributions were systematically monomodal whatever the ionic environment. In water, a D_h of 263 nm was observed that corresponded fairly well with TEM images. With salt addition, a significant shift toward smaller sizes (from 246 to 85 nm) was observed, showing a decreasing trend of D_h after heating.

Morphological Characterization of WPI Dispersions

The specific and combined effect of salt and heat on the morphology of WPI dispersions is highlighted in the TEM images presented in Figure 1. Without salt and at 30°C, well-separated roughly spherical nanoparticle structures presenting particle sizes below 70 nm were observed (Figure 1A-0%). At relatively low salt concentration (86 mmol/kg), small clumpy aggregates were noticed in addition to the well-separated spherical nanoparticles (Figure 1A-0.5%). When the NaCl concentration was increased up to 258 mmol/kg, the aggregates were clearer, denser, and larger (Figure 1A-1% and 1A-1.5%). Finally, particle aggregation clearly appeared in higher ionic environments (344 to 517 mmol/kg). A change in the structure of protein aggregates was observed, the aggregates being bigger and denser (Figure 1A-2% to 1A-3%).

Figure 1B shows the result of heat (90°C) on WPI dispersions. Without adding salt, compact/spherical aggregates took the form of white masses due to the

Table 1. Particle sizes (nm) obtained by static light scattering (SLS), dynamic light scattering (DLS), and from transmission electron microscopy (TEM) image analysis (mean of 3 independent measures)

NaCl (%)	At 30°C					At 90°C	
	Hydrodynamic diameter (DLS)		Particle size (SLS)			Hydrodynamic diameter (DLS)	Particle size (TEM) ¹
	First peak (nm)	Second peak (nm)	First peak (nm)	Second peak (nm)	Particle size (TEM) ¹		
0	5.8 ± 0.2	69.4 ± 5.9	— ²	—	≈70	263 ± 1.5	≈255
0.50	11.3 ± 0.8*	72.0 ± 2.6 ^{NS}	—	—	≈85 ^{NS}	246 ± 1.9 ^{NS}	≈228 ^{NS}
1.00	26.6 ± 3.8**	161.4 ± 14.8*	—	—	≈148**	182 ± 1.6*	≈115*
1.50	52.6 ± 16.3***	316.7 ± 11.3**	—	—	≈480**	118 ± 2.7**	≈100**
2.00	—	—	420 ± 6***	35,560 ± 13***	≈660***	101 ± 1.3**	≈100**
2.50	—	—	420 ± 9***	35,560 ± 7***	>1,000***	88 ± 6.2**	≈100**
3.00	—	—	420 ± 5***	35,560 ± 5***	>1,000***	85 ± 8.8**	≈100**

¹Mean of at least 10 particles observed in 3 different TEM images.²Dashes: not determined due to equipment limitation.*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ^{NS} $P > 0.05$ [comparisons between whey dispersion in water (control) and other concentration].

negative staining method used (Figure 1B-0%). The images also depict regions of excess stain, originating from the negative staining preparation method. These

structures present particle sizes around 260 nm. With addition of NaCl at relatively low concentration (86 mmol/kg), rather thinner and denser aggregates were

Figure 1. Transmission electron micrographs of whey protein isolate (WPI) dispersions after being heated at 30°C (A) and 90°C (B) and in variable NaCl environments (0, 0.5, 1, 1.5, 2, 2.5, and 3% salt). Imaging was performed at 24,000× magnification (scale bar = 200 nm). Each image is representative of at least 6 images observed.

observed (Figure 1B-0.5%). Upon increasing the salt concentration to 1.50% (258 mmol/kg), these spherical aggregates tended to be smaller and well dispersed (Figure 1B-1% and 1B-1.5%). Finally, at higher salt environments of 2 to 3% (344 to 517 mmol/kg), particles were characterized by a length less than 100 nm, less numerous, and more irregular in form (Figure 1A-2% to 1A-3%).

Rheological and Thermal Properties of WPI Dispersions

In Figure 2, WPI dispersions under variable NaCl ranges are plotted against the elastic modulus as a function of a heat ramp (30–90°C). A change in the viscoelastic properties of the dispersions was observed when subjected to thermal treatment. Without the addition of salt, the WPI dispersions showed nongelling behavior, presenting low values of G' throughout heating and holding a translucent appearance. In the presence of NaCl, the values of G' increased strongly between 75 and 80°C, showing a viscoelastic behavior and a plateau curve convincingly indicating an inflection point as temperature approached 88°C, demonstrating the formation of a continuous protein network. Furthermore, with addition of 0.50% NaCl, a sharp increase in the modulus was observed at 76°C, whereas

this behavior was noted at 80°C for 3% NaCl, showing a significant increase in the gelling temperature.

The effect of NaCl addition on the thermal properties of WPI dispersions was examined by μ DSC (Figure 3). In addition, their corresponding values of denaturation temperature are shown in Figure 4. The endothermic transition peaks indicated that structural changes were associated with temperature. The denaturation temperature of WPI dispersion without salt was 76.4°C, which shifted toward a higher temperature of 78.4°C at 0.50% salt. Increasing the NaCl concentration to 3% increased the denaturation temperature up to 80.2°C.

Protein Hydrophobicity

Analysis of the emission spectra (data not shown) of ANS-WPI dispersions in variable NaCl solutions with or without heating revealed a representative set of titration curves. Investigation of binding data allows the determination of values of $F_{\max}$ at saturating ANS concentration. The $F_{\max}$ is determined by the asymptotic value of the titration curve. The K_d of the fluorescent ANS-protein complex is determined as the ANS concentration required to obtain half the value of $F_{\max}$. From these 2 values, the PSH was calculated and indicated the binding average of the fluorescent marker to available hydrophobic sites of protein (Schmitt et al.,

Figure 2. Determination of rheological behavior (storage modulus, G' ; elastic component) of whey protein dispersions in variable NaCl environments (from 0 to 3%) from 30 to 90°C. Each curve is representative of 3 independent experiments.

Figure 3. Thermograms of whey protein dispersions in variable NaCl environments (from 0 to 3%). Each curve is representative of 3 independent experiments.

Figure 4. Denaturation temperature calculated from thermograms for whey protein isolate (WPI) dispersions in different NaCl environments. Results are the mean $\pm$ SD of 3 independent analyses.

Figure 5. Protein surface hydrophobicity (PSH) as a function of NaCl concentration for whey protein isolate (WPI) dispersions at 30 (▲) and 90°C (◆). Results are the mean $\pm$ SD of 3 independent analyses.

2009). The dependence of PSH on variable NaCl concentrations for heated and unheated samples is reported in Figure 5. When increasing the NaCl concentration or heat treatment, or both, the results are indicative of a decreasing trend for PSH values. The maximum values of PSH were observed around 1.6 and 0.40 (0% NaCl), whereas the lowest were found around 0.8 and 0.17 (3% NaCl) for unheated and heated dispersions, respectively.

Secondary Structure of WPI Dispersions

Curve Fitting and Peak Assignments. Second-derivative spectra were calculated from amide I and amide III bands. The frequencies of the bands obtained were used to define peak positions during the curve-fitting procedure. During the iteration process, the peak positions were allowed to shift. The positions were only slightly changed (less than 1.5 cm^{-1}). Optimal fits were obtained when the root mean square was in the range of 10^{-4} . Figures 6A and B present an example of the amide I and amide III regions fitted with a Gaussian line shape function for WPI in water at 30°C. Peak assignments of the amide I region were made using the results of former studies collected for globular proteins (Byler and Susi, 1986). The following structures were assigned: 1,700 to $1,660\text{ cm}^{-1}$, turns; 1,652 to $1,648$

cm^{-1} , α -helix; 1,648 to $1,642\text{ cm}^{-1}$, unordered; and $1,640$ to $1,620\text{ cm}^{-1}$, β -sheet. The same procedure was followed for the amide III region. Tentative assignments of amide III were also already done following Cai and Singh (1999): 1,330 to $1,295\text{ cm}^{-1}$, α -helix; 1,295 to $1,270\text{ cm}^{-1}$, β -turns; 1,270 to $1,250\text{ cm}^{-1}$, unordered; and 1,250 to $1,220\text{ cm}^{-1}$, β -sheet.

The amide I band was deconvoluted in 8 peaks (Figure 6A). The β -sheet structures were related to 3 bands. The band at $1,634\text{ cm}^{-1}$ is known to be strongly characteristic of amide groups involved in the extended β -sheet structure (Byler and Susi, 1986), whereas bands at 1,616 and $1,626\text{ cm}^{-1}$ exhibited low-frequency β -sheets. The low-frequency band at $1,616\text{ cm}^{-1}$ may have been caused by aromatic side chains ($\text{C}=\text{C}$ stretching vibrations) of the individual amino acids (Susi and Byler, 1988) and did not contribute much to the secondary structure. The relative intensity of the band at $1,634\text{ cm}^{-1}$ was the strongest, even though α -helix/random coils and turns were detectable. The band at $1,626\text{ cm}^{-1}$ was also assigned to β -sheet structure and, as evidenced by its low wave number position, was assigned to the formation of intermolecular β -sheet structure. The unordered structures were assigned to 2 bands ($1,641$ and $1,647\text{ cm}^{-1}$) and α -helix structures to the $1,655\text{ cm}^{-1}$ band. Finally, the presence of 1,666 and $1,681\text{ cm}^{-1}$ bands were related to β -turn structures. The

Figure 6. Example of Fourier transform infrared (FTIR) spectra obtained for whey protein dispersions in water at 90°C. Amide I (A) and amide III (B) region were fitted at peak positions obtained from second-derivative spectra followed by Gaussian curve fitting. Solid black squares represent experimental data; red (thick black in grayscale) curves represent the sum of the individual Gaussian components (β -sheets, unordered structures, α -helices, and β -turns). Color version available in the online PDF.

Table 2. Structural component (β -sheet, unordered, α -helix, and β -turn) percentage areas obtained for native whey protein dispersions in different NaCl environments measured from amide I and amide III at 30 and 90°C (mean of 3 independent analyses)

Structural component (%)	At 30°C						At 90°C					
	NaCl concentration (%)						NaCl concentration (%)					
	0	0.50	1.00	1.50	2.00	3.00	0	0.50	1.00	1.50	2.00	3.00
Amide I												
β -sheet	40.4	41.6 ^{NS}	38.9*	41.6 ^{NS}	41.6 ^{NS}	42.6 ^{NS}	62.3	59.7 ^{NS}	47.3*	44.7**	47.6*	47.6*
Unordered	21.6	21.5 ^{NS}	23.5 ^{NS}	24.1	24.3 ^{NS}	22.4 ^{NS}	10.6	8.0 ^{NS}	13.4*	14.0*	15.2**	14.8**
β -turn	16.0	14.9 ^{NS}	17.8 ^{NS}	14.5 ^{NS}	14.4 ^{NS}	14.5 ^{NS}	7.4	10.4 ^{NS}	14.9*	15.3**	18.7**	17.8**
α -helix	22.1	22.1 ^{NS}	19.8	19.8	19.8 ^{NS}	20.4 ^{NS}	19.7	22.0 ^{NS}	24.5 ^{NS}	26.0 ^{NS}	18.5 ^{NS}	19.9 ^{NS}
Amide III												
β -sheet	44.9	46.3 ^{NS}	45.3 ^{NS}	43.2 ^{NS}	45.8 ^{NS}	48.4*	71.1	58.7**	61.0*	65.1*	55.7**	53.0***
Unordered	22.5	20.0 ^{NS}	17.2*	22.4 ^{NS}	21.4 ^{NS}	18.4*	10.3	13.2*	13.9*	12.7*	17.4***	18.0***
β -turn	14.7	18.0*	19.7*	16.6 ^{NS}	16.4 ^{NS}	16.3 ^{NS}	5.3	17.5**	17.3**	12.3*	18.0**	17.8**
α -helix	17.8	15.7*	17.8 ^{NS}	17.7 ^{NS}	16.3 ^{NS}	16.9 ^{NS}	13.3	10.7 ^{NS}	7.8*	9.8 ^{NS}	8.9 ^{NS}	11.2 ^{NS}

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ^{NS} $P > 0.05$.

amide III band was deconvoluted in 5 peaks (Figure 6B). The maximum absorbance for β -sheets (2 bands) and α -helix (1 band) was around 1,241 cm^{-1} and 1,318 cm^{-1} , respectively. The wavenumbers at 1,261 and 1,284 cm^{-1} showed unordered and β -turn structures.

Quantitative Analysis. Areas calculated from the fitted spectra were considered to be proportional to the conformational elements that were assigned and are presented in Table 2. From amide I and III, no significant differences in structural percentages were noticed during NaCl increase for WPI dispersions without heating. Nevertheless, differences were observed between amide I and III results upon heating for the same salt concentration. For example, in water, the distribution in α -helix, β -sheet, β -turn, and unordered structures obtained was around 22, 40, 16, and 22% at 30°C and 20, 62, 7, and 11% at 90°C for amide I. For amide III, the percentages obtained were 18, 45, 15, and 23% and 13, 71, 5, and 10% for 30 and 90°C, respectively (Table 2). Nevertheless, these results were consistent with those found in the literature for globular proteins (Panick et al., 1999).

DISCUSSION

WPI Dispersions Size and Morphology with Combined Effect of NaCl and Heat

Dispersions in Water. Concomitant observations were obtained from TEM images and size analyses. Well-separated roughly spherical nanoparticles (around 70 nm) were observed by TEM and measured by DLS. Another population was also found by DLS with a D_h around 6 nm. These small particles were not visible on TEM images certainly due to the magnification used and may correspond to individual whey proteins. These

2 D_h are in agreement with values determined by Martinez et al. (2009) and Schmitt et al. (2007) for β -LA alone (corresponding to the first peak) and serum heat-induced milk proteins (corresponding to the second peak). The lack of aggregation can be related to the presence of substantial negative charge. Hence, strong electrostatic repulsions between molecules may prevent them from coming into close proximity (Baussay et al., 2004). For heated dispersions in water, the D_h value obtained by DLS was in agreement with values reported by others (Schmitt et al., 2011). This also coincides well with the particle size determination made from TEM images and assuming a complete protein denaturation and formation of clumps of big soluble spherical aggregates.

Dispersions in Low-NaCl Environments (<200 mmol/kg). The addition of sodium chloride in the system affected the size and morphology of the WPI aggregates. Low salt concentrations (86 mmol/kg) resulted in the formation of small clumpy aggregates (TEM images). Nevertheless, the D_h were not significantly different from those found in water. These discordances may be correlated to the important standard deviation found for the D_h . Aggregation is known to be strongly influenced by attractive and repulsive forces. The major forces favoring protein aggregation are hydrophobic and van der Waals forces, whereas electrostatic and hydration repulsions oppose this phenomenon. It seems that WPI started to aggregate above a critical salt concentration. Similarly, (Unterhaslberger et al., 2006) had already shown that when low amounts of NaCl were added (<60 mmol/kg), an insufficient number of chloride ions may have screened the positive charge of protein so that the preferential hydration forces were prevalent. In our concentrations, the electrostatic repulsion between protein molecules

may have been screened sufficiently and more chloride ions could bind to the protein surface so that the preferential hydration by water was overcome, leading to aggregation. It appeared also that at low ionic strength the primary aggregates associated mainly head to tail (Pouzot et al., 2005). This enabled the protein molecules to approach closely enough together to form clumpy aggregates. Conversely, the D_h and particle diameters observed by DLS/TEM of the heat-treated samples with or without salt presented a monomodal distribution showing a decreasing trend and totally differed from the particle size distribution of non-heat-treated dispersions. This phenomenon is a characteristic of an ongoing protein denaturation (Schokker et al., 1999). These findings can be explained by the second effect of ionic strength (i.e., salting-out effect of salts). At NaCl concentrations (≤ 200 mmol/kg), more chloride ions bind to the protein surface so that preferential hydration by water is overcome, leading to the observed denaturation and further protein aggregation coagulation phenomenon. Some other findings (Unterhaslberger et al., 2006; Nicorescu et al., 2008) also support our results, although it seems that it widely depends on co-solute, concentration, chemical environment, as well as the technological and operating conditions.

Dispersions in High-NaCl Environments (>200 mmol/kg). Increasing NaCl up to 258 mmol/kg resulted in an acceleration of WPI aggregation and the formation of denser aggregates. Dynamic light scattering and SLS measurements were in agreement with TEM images and the presence of aggregates were detected. From 258 to 517 mmol/kg, the electrostatic repulsions between protein molecules were screened more efficiently (i.e., the ionic strength was high enough to screen electrostatic interactions). These networks consisted of aggregated protein particles that are typically between 0.1 and 10 μm in diameter, hence forming a large number of aggregated protein particles within each particle, associated into particulate structures that have relatively large sizes (Langton and Hermansson, 1996; Verheul et al., 1998). It is worth noting that the contrast between particles and background in the TEM images was higher at these concentrations, depicting a denser structure of WPI aggregates. Such local densification and branching association of the protein aggregates in salt aqueous environment was reported by others for whey proteins (Pouzot et al., 2004). Upon heating the dispersions in higher salt concentrations (258–517 mmol/kg), particle sizes measured by DLS and TEM were well correlated and showed a similar decreasing trend. As already discussed, the likely explanation for smaller particles could be a combined salt/heat-induced denaturation process, leading into solid

aggregated gels at higher salt concentrations. Moreover, the size and microstructure of particles was insensitive to higher salt concentration (>200 mmol/kg). The transition from spherical/compact protein aggregates to linear ones upon salt/heat increase has already been reported for WPI dispersions (1% wt) at 85°C (Schmitt et al., 2007).

Rheological and Thermal Properties of WPI Dispersions

Gelling properties of WPI dispersions are significantly influenced by the concentration of NaCl present in solution (Mulvihill and Ennis, 2003). The lack of gelation in the absence of salt occurs because protein molecules have substantial negative charges; hence, a relatively strong electrostatic repulsion occurs between unfolded protein molecules, which prevents them from coming into close proximity (Bryant and McClements, 1998). It is virtually impossible to induce gelation by denatured proteins, in itself, as individual molecules must associate with one another to form a continuous network (Mulvihill et al., 1990). In the presence of NaCl, the G' increase is logical, as salt addition leads to a reduced intermolecular repulsion of unfolded protein molecules. Thus, it results in an increase in both the protein-protein interactions and their aggregation capacity, which finally contributes to a strong 3-dimensional protein network that contributes to a gel (Lorenzen and Schrader, 2006). It is noteworthy that higher G' values and maximum gel strength were observed at low NaCl concentrations. These results can be explained by the screening of negative charges carried by unfolded proteins, which in turn allowed an increase in strong protein-protein interactions in this salt range. A considerable increase in the gel firmness and stabilizing effect in the presence of 0.2 M NaCl was confirmed by others (Bertrand and Turgeon, 2007). Nevertheless, the addition of higher concentrations of NaCl (>200 mmol/kg) has shown negative influence on gel formation/firmness by decreasing protein unfolding (Boye et al., 1995) and increasing protein-solvent interactions (Sun and Arntfield, 2011). At higher NaCl ranges (>200 mmol/kg), Chantrapornchai and McClements (2002) observed a decrease in G' due to either an increase in size of insoluble heat-induced aggregates or a decrease in the number of protein-protein contacts.

By increasing the ionic strength, a significant increase in denaturation temperature for WPI dispersions was observed. Such a shift in denaturation temperature was due to structural rearrangements induced by NaCl. An identical increase in thermal stability has also been observed for whey proteins (Nicorescu et al., 2008) and

β -LG (Haug et al., 2009). Whey protein isolate dispersions are composed of 2 major proteins (β -LG and α -LA) presenting denaturation temperatures around 76 and 67°C, respectively (Ju et al., 1999). These temperatures are normally pH dependent. For example, the temperature of denaturation increased (85°C) for β -LG (Boye et al., 1997) and decreased (58°C) for α -LA (Bernal and Jelen, 1984) by lowering their pH. In our case, the pH of all systems remained stable. The shifts observed in denaturation (μ DSC) and gelling (rheometer) temperatures for WPI dispersions are well corroborated and showed that an effect of combined heat treatment and ionic strength may become a rate-limiting factor for their final functional properties.

PSH Index

The significant decrease in the PSH index with NaCl addition could be related to a lower availability of hydrophobic groups, certainly due to the formation of large aggregates. This evolution was characteristic of the presence of fewer hydrophobic sites available to ANS markers. This may be related to the fact that hydrophobic groups are buried inside the native structure of WPI molecules, so that with the salt-induced aggregation it is possible that their access to ANS probes is limited (Moro et al., 2001). Another explanation may be an interaction between hydrophobic sites of the proteins during salt-induced aggregation and a reduction in the number of available ANS-binding sites (Schmitt et al., 2007). Similarly, the PSH decrease observed for heated WPI dispersions may be due to an ongoing denaturation-aggregation phenomenon and structural collapse due to high temperature, resulting in limited availability of ANS-binding sites and, in turn, insoluble aggregates with decreased surface hydrophobicity. Some authors also observed a decrease in the PSH of whey proteins when increasing the temperature from 75 to 80°C (Carbonaro et al., 1996). It is worth noting that although a gradual decrease in PSH values occurred upon addition of NaCl under heated and unheated conditions, except with 1.5% NaCl, this decrement was limited and coincided well with TEM micrographs and summarized data.

Secondary Structure of WPI with Salt/Heat Augmentation

Up to now, the amide I spectral region was the most commonly used band because of its very strong signal but some recent results indicate that the amide III region gives a better prediction for protein secondary structures than does amide I (Cai and Singh, 2004;

Hussain et al., 2011a). This is because the amide I band suffers from an unstructured spectral contour and an overlap of the random coil and α -helix bands (Cai and Singh, 2004). In this work, the distributions of components within classes of structures are reproducible and the tendencies are exactly the same whatever the band analyzed. At room temperature (and pH around 6.6), the conformation (β -sheet, unordered, β -turn, and α -helix) of whey proteins was not appreciably altered by NaCl (from 86 to 517 mmol/kg). These results are corroborated by Boye et al. (1996). A similar effect of NaCl on β -LG secondary structure has been reported by others (Verheul et al., 1998; Kavanagh et al., 2000). Upon heat treatment without salt, β -sheet structures increased primarily at the cost of β -turn and unordered structures. This ongoing denaturation/aggregation process at elevated temperatures was also observed during rheological behavior study of WPI dispersion, as discussed earlier. These results are in agreement with those of Panick et al. (1999) for β -LG studied by spectroscopic and scattering techniques. Conversely, these results differs from those of Zhu and Damodaran (1994) who described a progressive decrease in β -sheets along an increase in unordered structures while studying heat-induced conformational changes in WPI with different heating times. This conflict may be attributed to the method adopted for heating proteins. Moreover, a salt increase (86 to 517 mmol/kg) tended to illustrate a stabilizing effect of NaCl (Boye et al., 1996; Verheul et al., 1998), suggesting an increase in thermal stability of WPI dispersions followed by a decrease in percentages of secondary structural elements. This increase in thermal stability was also observed by DSC analysis, showing that higher temperatures may be required to disrupt the native proteins structure at higher ionic strength.

CONCLUSIONS

This work demonstrated that gelation properties of whey protein depend on both salt level and how heat treatment is applied. Experimental data showed that during heat treatment and at low ionic strength, the gel elastic modulus increased and the strongest gel stiffness was achieved. Higher salt concentrations led to a weaker gel, whereas no gels at all were formed without salt. Additionally, NaCl addition along with dynamic heat treatment showed a stabilizing effect on the secondary structural elements of both amide I and III bands and higher denaturation temperatures. A morphological investigation showed a transition from spherical/compact protein aggregates to linear ones due to combined salt/heat effect. Moreover, by combining the results of DLS

and TEM with those of PSH, one may conclude that the number of hydrophobic sites available decreased significantly because of the formation of denser aggregates. For the first time, a combination of techniques with additional work on fundamental aspects of whey proteins was presented. These results add additional value and could validate other studies that independently made such observations. Careful adjustment of heat and NaCl concentration are necessary to effectively use WPI as a functional additive. Nevertheless, further work is required to understand sequential events occurring during salt- and temperature-induced unfolding and the aggregation process of whey proteins. This will be done in a future paper by use of FTIR combined with 2-dimensional correlation spectroscopy.

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