JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Ionic Strength and Denaturation Time on Polyethyleneglycol Self-Diffusion in Whey Protein Solutions and Gels Visualized by Nuclear Magnetic Resonance

Roxane Colsenet,[†] Olle Söderman,[‡] and François Mariette*,[†]

Process Engineering Technology Research Unit, Cemagref, CS 64426, 17 Avenue de Cucillé, 35044 Rennes, Cedex, France, and Physical Chemistry 1, Chemical Center, University of Lund, P.O. Box 124, S-22100 Lund, Sweden

Pulsed field gradient NMR spectroscopy was used to determine the poly(ethylene glycol) (PEG) self-diffusion coefficient (D_{PEG}) as a function of NaCl concentration (C_{NaCl}) and denaturation time (t_D) in whey protein solutions and gels. D_{PEG} in the gel decreased with increasing C_{NaCl} concentrations and increased with increasing t_D; the increase ceased for all PEGs when the gel was fixed. This increase was more pronounced for the 82250 g/mol PEG than the 1080 g/mol PEG. Moreover, the diffusion coefficient of nonaggregated whey protein was measured and an increase for longer t_D was also observed. Scanning electron microscopy images and ^1H spectra demonstrated that D_{PEG} were related to the structure changes and to the percentage of β-lactoglobulin denaturation.

KEYWORDS: Whey proteins; denaturation time; NMR; diffusion; ionic strength; SEM; gels

INTRODUCTION

Many food proteins are known to form gels when subjected to heating. An understanding of the relationship between the physicochemical conditions under which gels form and the accompanying gel characteristics is of major importance to optimize industrial food processing. This is particularly true for whey proteins, which are globular proteins composed of β-lactoglobulin (β-lg), α-lactalbumin (α-la), immunoglobulins (Ig), and bovine serum albumin (BSA). These proteins are widely used as food ingredients, because they are highly nutritious and possess valuable functional properties, the most important being their gelling properties (*1*). Three phenomena are (often simultaneously) involved in aggregation of globular proteins: conformational changes, chemical reactions, and physical interactions. On heating, whey proteins will partly unfold. Nonpolar groups are exposed in this denaturation step, and in the case of β-lg and BSA, the free sulfydril groups become reactive. The second step is a rearrangement of the denatured molecules leading to aggregation and network formation. As reported in the literature, the structure and properties of whey protein gels vary with the extent of unfolding, the type and kinetics of the aggregation process, and the nature of the interactions (*2*). Moreover, at the time a gel is formed (the gel point), only a fraction of the whey proteins has aggregated (*3, 4*), the magnitude of this fraction and the gel point depending on medium composition and heating conditions. After the gel point, more proteins are incorporated in the gel network while gel rigidity increases (*3*).

Changing solution pH, salt concentration, type of salt, and heating conditions alter the gelling properties of whey proteins and the gel structure formed. At pH values sufficiently far from the isoelectric point of the proteins and at low ion concentrations, the unfolded proteins tend to remain separate due to the electrostatic repulsive forces between them. Upon addition of salt, these repulsive forces are screened, and the protein molecules can aggregate and form a gel (*5–7*). For example, transparent gels with a fine-stranded structure are formed when there is considerable electrostatic repulsion between the proteins (at low ionic strength <0.1 M). Under conditions of reduced electrostatic repulsion (at high ionic strength), white gels are formed with a particulate structure (*8*). Rheological parameters have demonstrated that the gels become more brittle as the salt concentration increases and that the firmness of the gel increases significantly, showing that the thicker protein strands form a more rigid matrix (*5, 9, 10*). Stading et al. (*8*) have produced scanning electron microscopy (SEM) images of β-lg gels (10 g/100 g) at different heating rates. Gels formed at a fast heating rate (t_D = 12 min) consisted of a homogeneous network, the strands being formed by evenly sized spherical particles linked like a flexible string of beads. At a slow heating rate (t_D = 10 h), the network had larger pores. On the other hand, little information is available on transport properties in whey protein gels.

Few studies have investigated diffusion in whey protein gels related to the structural changes in the gels. Le Bon et al. (*11*) measured β-lg self-diffusion coefficients in solutions (0–20 g/100 g) by varying the salt concentration (0.003 and 0.1 M). They found that the effects of electrostatic interactions on β-lg self-diffusion coefficients were weak. Beretta et al. (*12*) measured the β-lg self-diffusion coefficients in ammonium

* To whom correspondence should be addressed. Tel: 33(0)223482121. Fax: 33(0)223482115. E-mail: Francois.Mariette@cemagref.fr.
† Cemagref.
‡ University of Lund.

Table 1. Composition of Whey Protein Powder Per 100 g of Powder

	g for 100 g of whey protein powder
total solids	95.22
total nitrogen matter	14.65
total protein matter	91.56
lactose	0.5
calcium	0.509
potassium	0.234
magnesium	0.067
phosphorus	0.192
sulfur	1.329
α-lactoglobulin	54.1
β-lactalbumin	12.4
pure proteins in total solids	96.16

Table 2. Percentages of β-lg and α-la Denaturation of Whey Protein Gels (29.35 g/100 g) with Thermal Denaturation of Whey Protein Solution in a Water Bath at 70 °C for Various t_D (NaCl = 0.1 M)

t_D (min)	% of denaturation	
	β-lg	α-la
30	57.2	100
45	65.3	100
60	72.9	100
90	78.4	100
700	100	100

sulfate solutions for ionic strength between 0.6 and 6 M at various protein concentrations (from 0.1 to 3 g/100 g). They observed a decrease in β-lg diffusion with increasing protein concentrations and increasing ionic strength.

Croguennoc et al. (*13*) measured the β-lg self-diffusion coefficient in β-lg solutions (17.9 g/100 g). β-Lg self-diffusion was found to increase with heat treatment because the friction caused by the immobile structure of the gel is much less than that caused by mobile native proteins.

The principal probes used to study diffusion in gels are dextran or poly(ethylene glycol) (PEG). Croguennoc et al. (*13*) reported the only study on probe self-diffusion coefficients in β-lg solutions. They measured the dextran self-diffusion coefficients for various dextran molecular masses and denaturation times between 0 and 1000 min. The dextran self-diffusion coefficients decreased after heat treatment due to the friction with aggregated β-lg. In a previous study, probe size, protein concentration, and gelation effects were studied on PEG diffusion in whey protein solutions and gels (*14*). One of the results of this study was an increase in PEG self-diffusion coefficients with solution/gel transition after thermal treatment of 30 min. This increase was particularly noticeable for whey protein concentrations above 20 g/100 g. These results were explained by the creation of a network with more space, the aggregation of proteins being observed on SEM images. Phillies and Rouse models were used to describe the findings involving a deformation of the PEG chain to an ellipsoidal conformation. This deformation was more pronounced in protein solutions as compared to protein gels due to the creation of large voids with gelation.

In continuation of this previous (*14*) work, this paper presents an investigation of the effects of ionic strength (NaCl concentration) and denaturation time (heating time) at neutral pH on PEG diffusion in whey protein solutions and gels. PEG self-diffusion coefficients were measured by pulsed field gradient NMR spectroscopy (PFG-NMR), and SEM was used to observe the different gel structures. The variations of the PEG self-diffusion coefficients are discussed in terms of the structural changes in the protein gels as observed in SEM images.

MATERIALS AND METHODS

Materials. Whey protein powder (INRA, Rennes, France) was used. The powder composition is summarized in **Table 1**, and the percentage of protein denaturation is presented in **Table 2**.

Whey protein powder analyses were performed by SOREDAB laboratories (La Boissière Ecole, France). The PEG polymers [H(OCH₂-CH₂)]ₙOH (where *n* is the number of repeating units) were supplied by Polymer Laboratories (Marseille, France) with different average molecular weights (M_w = 1080 and 82250 g/mol) and low polydispersity indices (1.01 and 1.02, respectively, as indicated by the suppliers). All polymers were used without further purification. The

D₂O (purity above 99.8%) used for mixing the samples was purchased from Dr. Glaser (Basel, Switzerland). Sodium azide NaN₃ (Merck, Darmstadt, Germany) and NaCl were used without purification.

Sample Preparation. Rehydration of the whey protein powder was performed at room temperature with D₂O/NaCl solution (0.1−2 M). The use of D₂O reduces the interference between proton peaks of water and PEG and improves the accuracy of the PEG peak intensity values. Sodium azide was added (0.02% w/w) to each solution to prevent bacteria development.

For convenience, PEG was added at solutions (0.1% w/w) for each average molecular weight. The pH was measured, and no changes were observed between the samples (pH = 6.8 for 32 g/100 g whey protein solution with PEG 82250 g/mol).

Whey protein gels were prepared from whey protein solutions by thermal aggregation. The solutions were transferred to 5 mm NMR tubes (~1 mL) and kept in a water bath at 70 °C for different denaturation times, t_D (30−720 min), and then cooled to 20 °C. Water evaporation was checked by weighing NMR tubes before and after gelation. No significant loss of water content from the gel was observed.

NMR Self-Diffusion Measurements. The measurements were performed on a 200 MHz Bruker spectrometer, and some complementary measurements were performed on a 400 MHz Bruker spectrometer, both equipped with a field gradient probe. No significant variations were observed between the two spectrometers. Pulsed gradient spin−echo (PGSE: 90° − t_1 − 180° − t_1 − echo) (*15*) and pulsed gradient stimulated-echo (PGSTE: 90° − t_1 − 90° − $t2$ − 90° − t_1 − echo) (*16*) sequences were used to measure the PEG self-diffusion coefficient.

All of the measurements were conducted at 20 ± 0.1 °C. The gradient strength g used in this study ranged between 1.07 and 9.63 T/m. The diffusion coefficients for the two sequences were obtained using:

$$\frac{I(\delta,\Delta,g)}{I_0} = \exp[-kD] \qquad (1)$$

where $k = \gamma^2 g^2 \delta^2 (\Delta - \delta/3)$ and $I(\delta,\Delta,g)$ and I_0 are the echo intensities in the presence of pulsed gradients of strength g and the absence of gradient pulses, respectively. The length of the gradient pulse was δ, Δ was the distance between the leading edges of gradient pulses, τ was the time between the 90° and the 180° pulses, T_2 was the transverse relaxation time, γ was the gyromagnetic ratio (for protons, $\gamma = 26.7520 \times 10^7$ rad T⁻¹ s⁻¹), and D was the self-diffusion coefficient. In agreement with this equation, a semilogarithmic plot of echo intensities vs k is known as a Stejskal−Tanner plot. Equation 1 was used to calculate the PEG self-diffusion coefficient (D_{PEG}) with the intensities of the peak at 3.6 ppm.

A total of 16 or 32 scans was collected with PGSE and PGSTE sequences with a recycle delay of 1 s. Δ was adjusted to have the same diffusion pathway for each PEG, in accordance with the Einstein equation $z = (2D_{PEG} \Delta)^{1/2}$, with $z \sim 2 \mu m$. In this case, the distance covered by the PEG is greater than the whey protein diameter. For example, the value of the hydrodynamic radius of β-lg, which represents 54.1% of the whey protein powder, is in the range from 2 to 4 nm (*11, 12, 17, 18*).

D_{PEG} in pure D₂O at 20 °C was presented in a precedent study (*19*). PEG hydrodynamic radii (R_h) were calculated from D_0 and the Stokes−Einstein equation and were found equal to 1.18 and 13.39 nm for 1080 and 8250 g/mol PEG, respectively.

An example of a proton spectrum obtained for a diffusion measurement of 8500 g/mol PEG in a whey protein gel (35 g/100 g) with the

Polyethyleneglycol Self-Diffusion in Whey Protein

J. Agric. Food Chem., Vol. 54, No. 14, 2006 **5107**
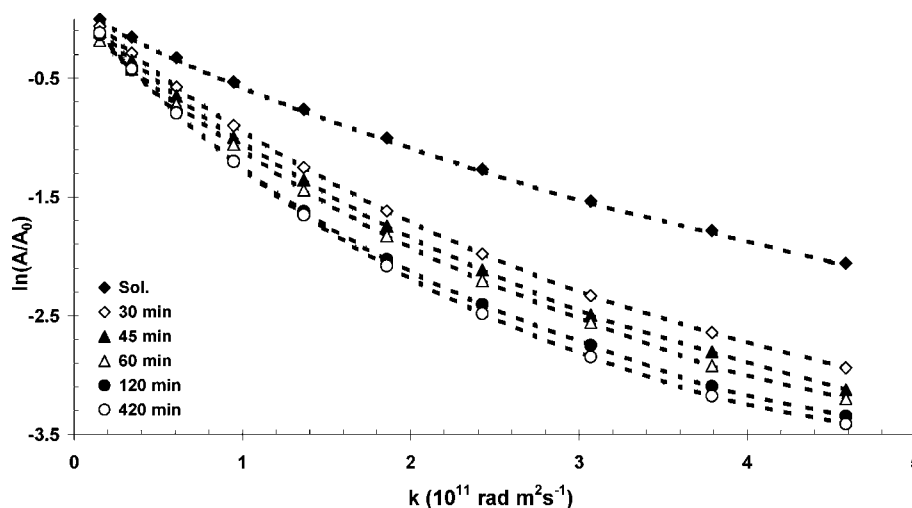
Figure 1. Stejskal–Tanner plots of the area (between 1 and 4 ppm) of whey protein signals (29.13 g/100 g) for various t_D. Dashed lines represent fits with the polydispersity model (eq 4) with $M_w/M_n \sim 1.2$ for all treatments.

PGSTE sequence was presented in a previous study (*14*). The protein signals were due to mobile proteins. The whey protein powder was composed of a mix of proteins of various sizes: β-lg (18400–36900 g/mol), α-la (14200 g/mol), Ig (160000 g/mol), and BSA (69000 g/mol) (*1*). The protein signals had contributions from several components, with the principal component resulting from β-lg diffusion in the majority in the powder. The protein self-diffusion coefficients were measured from the protein signal area between 1 and 4 ppm, and the data were analyzed by using the method of cumulants (*20, 21*). In terms of second-order cumulant expansion, the attenuation of the echo signal can be expressed as:

$$\ln\frac{I}{I_0} = -k\langle D \rangle + \frac{1}{2}k^2\langle D \rangle^2 [1 - (M_w/M_n)^{-4/5}] \quad (2)$$

where $\langle D \rangle$ is an average diffusion coefficient and M_w/M_n is the polydispersity index. For whey protein diffusion, M_w/M_n represents the variability of the whey protein self-diffusion coefficients depending on the protein size.

All of the data processing was performed with Matlab or Tablecurve software. Monte Carlo simulations were used for error calculations (*22*), with 100 iterations.

SEM. Small cubes of the gels (5 mm × 5 mm × 5 mm) were cut out and immersed in 2.5% v/v glutaraldehyde at room temperature for 48 h and then rinsed thoroughly three times for 10 min with distilled water, after which they were immersed in 0.2% v/v OsO_4 overnight at room temperature. The samples were rinsed several times with distilled water before being dehydrated in a graded ethanol series [10–30–50–70–80–90–95–100% (v/v)] in 20 min steps. Samples were then critical point dried through CO_2 in a critical point drier (CPD 010, Balzers Union Ltd., Liechtenstein). Dried samples were fractured, mounted onto specimen stubs, gold coated, and analyzed microscopically using a scanning electron microscope (JEOL JSM 6301F) operated at an acceleration voltage of 9 kV. The images were produced by CMEBA (Rennes, France).

RESULTS

Thermal Treatment Effect. PEG self-diffusion coefficients (1080 and 82 250 g/mol) were measured in whey protein solutions (32.17 and 29.13 g/100 g) heat-treated with 0.1 M NaCl at 70 °C for various t_D values. High protein concentrations were chosen because the effects of the gelation were clearly visible above a protein concentration of $\sim$20 g/100 g (*19*). For PEG, the Stejskal–Tanner plots were linear for all values of the denaturation time t_D (results not shown). **Figure 1** presents the Stejskal–Tanner plots of whey proteins for different t_D values in whey protein solutions and gels (29.13 g/100 g). The whey proteins were a mixture of several proteins of different
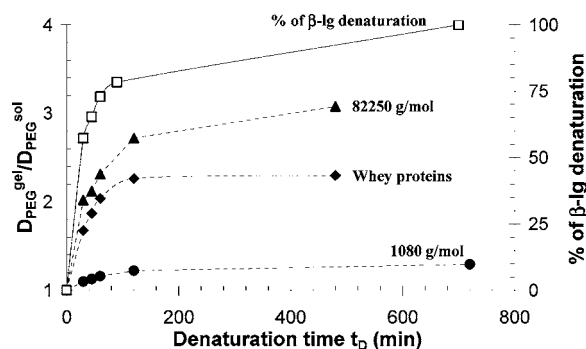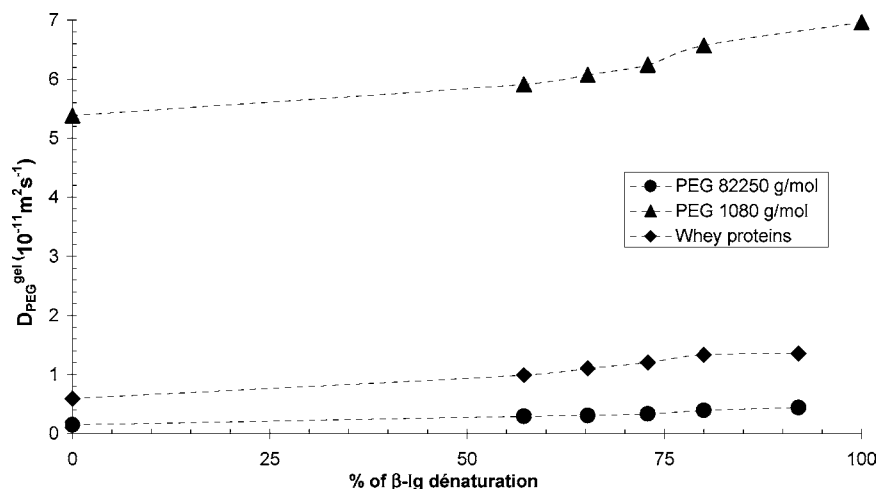

Figure 2. Self-diffusion coefficient of PEGs and whey proteins in whey protein solutions and gels (32.17 and 29.13 g/100 g) normalized by self-diffusion coefficients in corresponding solutions ($t_D = 0$) vs t_D ($C_{NaCl} = 0.1$ M and $T_D = 70$ °C) measured at 20 °C. The % of β-lg denaturation is presented on the second axis vs t_D.

sizes and different self-diffusion coefficients, and thus, the Stejskal–Tanner plots were not linear. Attenuation data were analyzed by using the method of cumulants (*20, 21*), and the value of M_w/M_n was found to be around 1.2 for all t_D values.

Proton NMR spectra of β-lg (*13*) solutions heated at different t_D values showed a decrease in all of the protein peaks with increasing t_D values. The authors concluded that the decrease in native proteins observed indicates that only the signal of nonaggregated β-lg can be seen. They confirmed that the smallest aggregates already contained about 100 proteins ($R_h \sim 30$ nm) and therefore that their mobility was very much reduced. In this study, the whey protein self-diffusion coefficients therefore measured corresponded to native proteins or denatured proteins but not to reticulated proteins.

Figure 2 shows the reduced self-diffusion coefficients for 1080 and 82 250 g/mol PEG ($D_{nPEG} = D_{PEG}^{gel}/D_{PEG}^{sol}$) and non-reticulated whey proteins ($D_{nprot} = D_{prot}^{gel}/D_{prot}^{sol}$) in protein gels in relation to t_D at corresponding whey protein concentrations. D_{PEG}^{sol} and D_{prot}^{sol} were the diffusion coefficients in whey protein solution for PEG and whey protein, respectively. On the same figure, the percentage of β-lg denaturation was plotted as a function of t_D.

Diffusion of 1080 and 82 250 g/mol PEG increased when the denaturation time increased over the whole range of t_D. This increase was more pronounced for the 82250 g/mol PEG ($R_h = 13.39$ nm) than the 1080 g/mol PEG ($R_h = 1.18$ nm). For the smallest PEG (1080 g/mol), two distinct steps were observed

Figure 3. Self-diffusion coefficient of PEGs and whey proteins in whey protein solutions and gels (32.17 and 29.13 g/100 g) ($C_{NaCl} = 0.1$ M and $T_D = 70$ °C) vs the % of β-lg dénaturation at varying denaturation time t_D.

as follows: a large increase between 0 and 120 min (+22.1% in 2 h) and then a small increase between 120 and 720 min (+5.9% in 10 h). A D_r value of 82250 g/mol PEG showed a greater increase between 0 and 120 min (+171.8%) and between 120 and 240 min (+13.2%).

The protein self-diffusion coefficients showed the same variations as the PEG diffusion when t_D was changed. Whey proteins diffusion increased with t_D between 0 and 120 min (+126%), and a plateau was obtained between 120 min and 4 h (+1.48%).

All of the diffusion coefficients were in excellent correlation with the % of β-lg denaturation according to t_D, for which we observed a strong increase between 0 and 90 min (78% denatured) and a plateau between 90 and 700 min (+21.6% denatured).

Figure 3 shows the self-diffusion coefficient of PEGs (1080 and 82 250 g/mol) and whey proteins in whey protein solutions and gels (32.17 and 29.13 g/100 g) according to the percentage of β-lg denaturation at varying denaturation time t_D. The self-diffusion coefficients present a variation close to the linearity with an increasing β-lg denaturation percentage.

Power Law Dependence. A simple power law gives a description of the solute diffusion coefficient vs molecular weight:

$$D_{PEG} = A \cdot M_w^{-\alpha} \tag{3}$$

with A being a preexponential factor and α a characteristic exponent.

In fact, this equation is often used to describe the self-diffusion of polymer chains with α varying from 0.55 for dilute systems (23) to 2.5 in concentrated systems (24), where the change is the value of α brought about by the transition from Rouse-like to reptation-like diffusion. Equation 3 was applied to PEG diffusion in whey protein solutions and gels in a previous study (14). The plots of D_{PEG} and D_{prot} vs PEG and whey protein molecular mass (M_w) in whey protein gels for each denaturation times are given in **Figure 4**. PEG diffusion values in solutions and gels at $t_D = 30$ min were extracted from the previous study (14). The findings were coherent by assuming a whey protein molecular mass of 16000 g/mol. This value was close to the β-lg molecular mass (18400 g/mol) by taking into account the small number of experimental points. The exponent α decreased when t_D increased.

The microstructure of whey protein gels (32.24 g/100 g) was studied at three t_D (30−90 and 480 min) with $C_{NaCl} = 0.1$ M
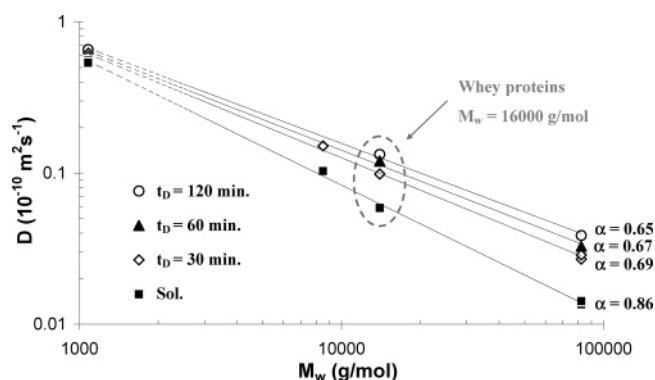

Figure 4. Power law representation of PEG self-diffusion coefficients in D_2O and whey protein gels vs PEG molecular mass for various whey protein concentrations.

and heat treatment at 70 °C. The images are presented on **Figure 5a−c** with a magnification 50000×.

The gel formed at the lowest t_D (30 min) had a relatively homogeneous structure composed of small compact aggregates. At 90 min, the aggregates appeared biggest and less dense; moreover, larger pores were detected. When t_D was increased to 480 min, a fine-stranded gel microstructure with more open pores was observed. SEM images were in accordance with the PEG self-diffusion coefficients. When t_D increased, more proteins were incorporated into the gel matrix, decreasing the aqueous phase viscosity and creating larger voids (**Figure 5c**), causing the diffusion coefficients to increase. Moreover, these observations were in agreement with a more transparent gel when the denaturation time increased.

NaCl Effect. Figure 6 shows the proton spectra of whey protein gels at 32 g/100 g measured at different NaCl concentrations (0.2−2 M). The spectra show a collection of peaks between 1 and 8 ppm due to the various protein protons. The PEG peak was observed at 3.6 ppm, and the water peak was observed at 4.73 ppm. All of these spectra showed good correlation with those reported in the literature (11, 13), and the protein signals observed correspond to mobile, native, or denatured proteins but not to aggregated proteins as previously demonstrated (13). The proton peaks of proteins (i.e., at 7.2 ppm) showed a loss of resolution in whey protein gels with an increase in NaCl concentration, reflecting a decrease in molecular mobility. It proved to be difficult to measure the protein self-diffusion coefficients accurately with the sequence parameters used. Therefore, only PEG diffusion is discussed below.
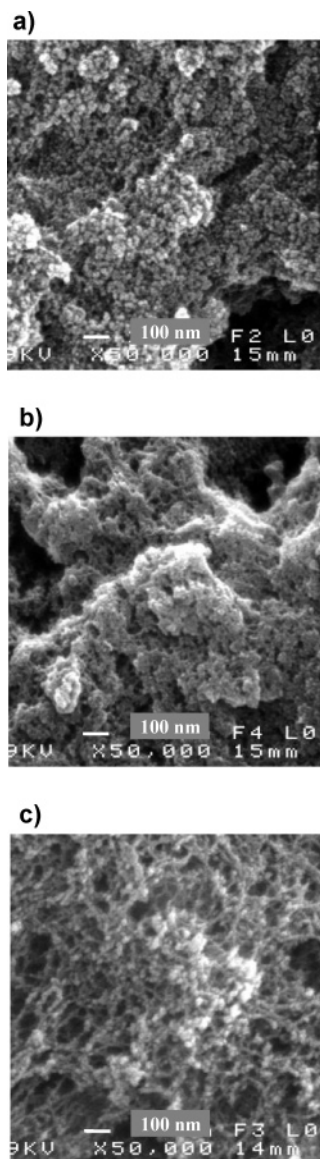
Figure 5. SEM images of whey protein gels (32.24 g/100 g) at various t_D (a) 30, (b) 90, and (c) 480 min with C_{NaCl} = 0.1 M and T_D = 70 °C (magnification, 50000×).

The self-diffusion coefficients of 1080 g/mol PEG in whey protein solutions and gels were measured at 20 °C by varying the NaCl concentration at two whey protein concentrations (6.75 g/100 g and 32.17 g/100 g). Examples of Stejskal−Tanner plots are presented for PEG diffusion in gels at 32.17 g/100 g in **Figure 7**. All of the Stejskal−Tanner plots were linear for the two concentrations at the length probed (∼2 μm). The gel was therefore homogeneous over this length scale.

D_{PEG} was measured in D_2O added to the corresponding NaCl concentration (D_0) and was normalized by these values in order to take into account the change in viscosity of the water phase induced by NaCl.

Figure 8 shows $D_r = D_{PEG}/D_0$ of 1080 g/mol PEG in whey protein solutions and gels (6.75 g/100 g) according to NaCl concentration.

An appearant decrease in D_r in solutions and gels was observed with increasing NaCl concentrations; however, this decrease was not significant. Similarly, the difference between the diffusion in solution and gel for a given NaCl concentration was negligible. The increase in ionic strength in the case of low whey protein concentration solutions had no effect on 1080 g/mol PEG diffusion.
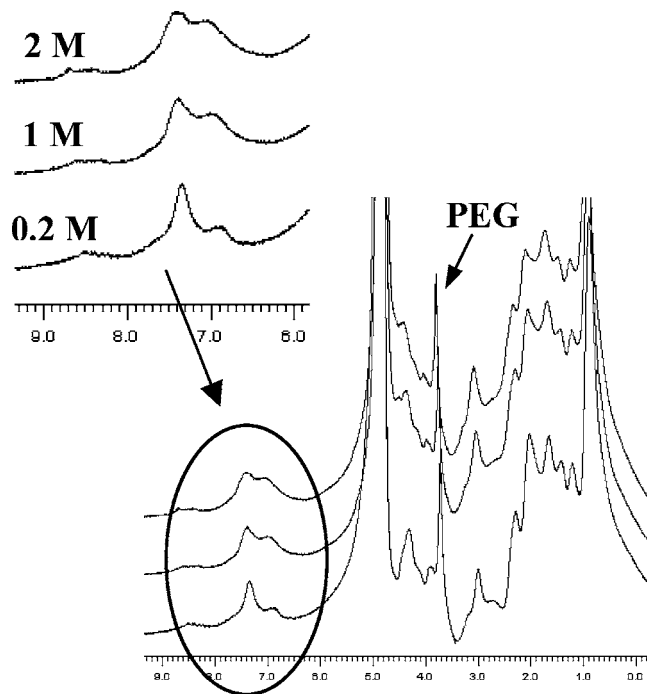
Figure 6. ^1H spectra of whey protein gels (32.17 g/100 g) at various NaCl concentrations.

The effects of ionic strength on PEG diffusion were observed in a more concentrated solution. **Figure 9** shows a D_r of 1080 g/mol PEG in whey protein solutions and gels (32.17 g/100 g) for various NaCl concentrations. The diffusion time Δ was 20 ms, and the root means square displacement was of order 1.5 μm. All of the Stejskal−Tanner plots (data not shown) were linear on this scale. The first findings were the absence of effects of NaCl on PEG diffusion in whey protein solutions since D_r was constant on the entire range of NaCl concentrations. On the other hand, D_r in gels decreased when NaCl concentration increased from 0 to 0.5 M; the self-diffusion coefficient value decreased from 5.94 × 10^{-11} to 4.78 × 10^{-11} m^2 s^{-1}, and then, a plateau was observed between 0.5 and 2 M. PEG diffusion in gels was faster than in the solutions over the entire range of NaCl concentrations. The difference was significant with no salt added (5.40 × 10^{-11} m^2 s^{-1} in solution against 5.94 × 10^{-11} m^2 s^{-1} in gel), decreased between 0 and 0.5 M, and was stable for higher NaCl concentrations (4.67 × 10^{-11} m^2 s^{-1} in solution for 4.78 × 10^{-11} m^2 s^{-1} in gel).

The microstructure of whey protein gels was studied by SEM at 32.24 g/100 g, and NaCl concentrations (C_{NaCl}) were 0.1 and 2 M. The thermal treatment conditions were 70 °C for 30 min in a water bath. Under these experimental conditions, the macroscopic appearance of the gels was opaque and white. **Figure 10** shows the SEM images made with C_{NaCl} = 0.1 and 2 M with 20000× (**a**, **b**) and 50000× (**c**, **d**) magnifications. The gel network is clearly apparent, and a three-dimensional view was visualized. The large focus depth made it possible to obtain a good view of the coarse network with C_{NaCl} = 0.1 M and clearly showed the open structure of the gel (**Figure 10c**).

At C_{NaCl} = 2 M, the images showed a clear change in microstructure. **Figure 10b,d** shows the flat surface of the fine-stranded network, which has ruptured at the particles, resulting in ditches in the fracture plane. The network was denser as compared with the images at C_{NaCl} = 0.1 M. The less porous structure of the gel with C_{NaCl} = 2 M showed good correlation with a decrease in 1080 g/mol PEG self-diffusion coefficient.
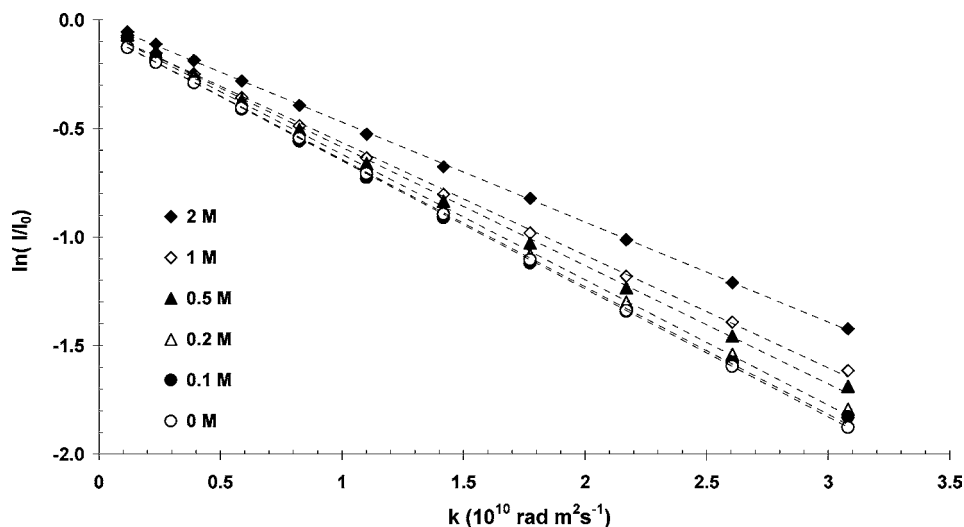
Figure 7. Stejskal–Tanner plots of 1080 g/mol PEG in whey protein gels (32.17 g/100 g) for various NaCl concentrations.
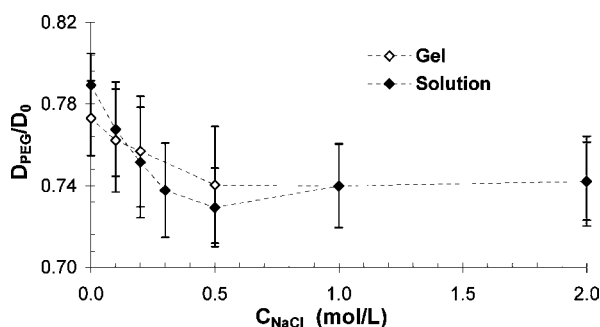

Figure 8. D_r of 1080 g/mol PEG in whey protein solutions and gels (6.75 g/100 g) vs NaCl concentrations normalized by self-diffusion coefficient of PEG in D_2O + NaCl at the corresponding concentrations at 20 °C measured with 200 MHz spectrometer and PGSE sequence.
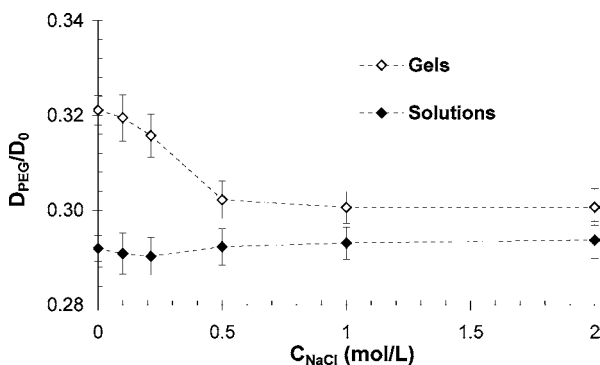

Figure 9. D_r of 1080 g/mol PEG in whey protein solutions and gels (32.17 g/100 g) vs NaCl concentrations at 20 °C measured with 200 MHz spectrometer and PGSE sequence. Normalization was obtained by self-diffusion coefficient of PEG in D_2O + NaCl at the corresponding concentrations.

DISCUSSION

These results show that PEG self-diffusion coefficients were always higher in whey protein gels than in solutions whatever the experimental parameters of gelation (variations of C_{NaCl} or t_D). However, the magnitude of the increase depended on the PEG size and the physicochemical conditions of the gelation.

The 1080 g/mol PEG (R_h = 1.18 nm) was less sensitive to changes in the structure induced by aggregation than 82250 g/mol PEG (R_h = 13.39 nm). When the probe size increased, the PEG self-diffusion coefficient became more sensitive to
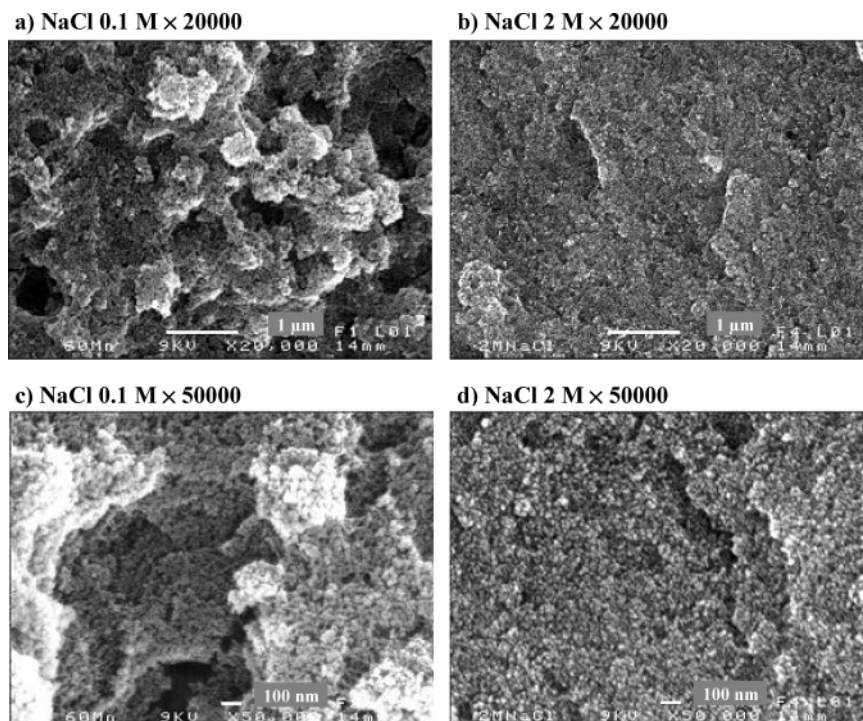
structure changes induced by thermal aggregation of whey proteins. The nonreticulated whey proteins ($R_h \sim 2$ nm) showed an increase of around +126% between 0 and 120 min, intermediate between the increase in the 1080 and 82250 g/mol PEG. The effects of t_D increased when the size of the diffusing molecules increased: Large molecules seemed to be more affected by gelation than smaller molecules, most likely because the obstruction effects are smaller in solution for small molecules and the effects of a more space-spanning structure on diffusion are less significant. These observations were well-described by a power law. The increase of α with t_D suggested an increase of intraparticle distances in the gels.

At the gel point, the primary spatial structure of the gel was fixed and the amount of protein aggregated determined this spatial structure. After the gel point, more proteins were incorporated in the gel network and gel rigidity strongly increased. The large-scale structure of whey protein was related to the electrostatic repulsion and the stability/solubility of the protein molecules and aggregates. When whey proteins "fitted" in the gel network, the viscosity of the aqueous phase determined by the nonreticulated whey protein concentration decreased.

The plateau obtained for PEGs and proteins between 120 min and 8 h demonstrated that the gel had reached a stable structure after 2 h of thermal treatment and the thermal aggregation had no further effect on PEG diffusion. This showed excellent correlation with the percentage of β-lg denaturation since at t_D = 120 min almost all whey proteins were denatured.

An increase in protein diffusion with an increase of denaturation time has already been reported by Croguennoc et al. (*13*) for the diffusion of native β-lg. At a concentration of 17.9 g/100 g and thermal treatment at 70 °C, the self-diffusion coefficient increased from 2.3 × 10^{-11} to 4.8 × 10^{-11} m^2 s^{-1} between 1 and 20 min and stabilization occurred between 20 and 1000 min. To explain these variations, frictions of native proteins with other native proteins and with larger aggregates and the gel were considered. However, friction with other native proteins decreased in proportion to the fraction of residual native protein and friction with large aggregates and the gel was less than with native proteins because the increase in pore size was larger than the protein size. As a consequence, the increase in protein self-diffusion coefficient reflected the decrease in the fraction of native proteins. This is in full agreement with the results of our study.

Croguennoc et al. (*13*) studied dextran diffusion (62000, 250000, and 1700000 g/mol) in 7 g/100 g of β-lg at different t_D values (between 0 and 16 h) and reported that $D_{dextran}$

Polyethyleneglycol Self-Diffusion in Whey Protein

J. Agric. Food Chem., Vol. 54, No. 14, 2006 **5111**

a) NaCl 0.1 M × 20000

b) NaCl 2 M × 20000

c) NaCl 0.1 M × 50000

d) NaCl 2 M × 50000

Figure 10. SEM images of whey protein gels (32.24 g/100 g) with t_D = 30 min at 70 °C for two NaCl concentrations and two magnifications: (a–c) C_{NaCl} = 0.1 M and (b–d) C_{NaCl} = 2 M.

decreased for all dextrans after heat treatment due to friction with aggregated β-lg. The reduction was more pronounced when the dextran size increased (D_r = 0.9, 0.5, and 0.4 at 30 min, respectively). Once the protein gel was formed (at $t_D \sim 100$ min), $D_{dextran}$ remained approximately constant. The decrease in dextran diffusion could be explained by interactions between dextran and β-lg. Zhang et al. (*25*) showed by turbidity measurements that a high concentration of dextran accelerated the formation of β-lg aggregates whereas a low concentration retarded it. They suggested that dextran induced steric hindrance, which probably excluded β-lg. Moreover, the dextran concentration (∼4%) was higher than the PEG concentration used here (∼0.1%) and increased the possibility of entanglement of the polymers with the proteins.

Increasing NaCl concentrations created a denser network, as observed on SEM images. Bryant et al. (*10*) showed by rheological measurements that increasing the salt concentration resulted in more extensive shielding of negatively charged whey protein aggregates, resulting in creation of larger aggregates (*26, 27*). The structure changes at low whey protein concentrations, due to increased NaCl concentrations, had no effect on 1080 g/mol PEG diffusion in solutions and gels. On the other hand, at high whey protein concentrations, diffusion was always faster in gels than in solutions, whatever the NaCl concentration. The plateau observed between 0.5 and 2 M indicated that all of the NaCl had reacted and assumed a stabilization of the gel network structure.

Ikeda et al. (*17, 28*) suggested that gelation with increasing ionic strength does not involve fundamental changes in the aggregation mechanism, but the size of the elementary particles appeared to be larger than those formed in the absence of NaCl. The increase in NaCl created larger aggregates of whey proteins; on SEM images, a more open structure was observed at C_{NaCl} = 2 M as with C_{NaCl} = 0.1 M, but a very dense structure occurred with no distinct pores and shorter distances between the junctions.

Langton and Hermansson (*29*) and Verheul (*4*) suggest that the addition of salt (NaCl) has an effect on spatial structure

similar to that of lowering pH. In conditions of low ionic strength (NaCl ∼ 0.05 M), gels from globular proteins are transparent and have a fine-stranded structure, whereas they are opaque with a coarse, lumpy, particulate structure if the ionic strength is increased to 0.2 M NaCl. The network structure in a heat-induced globular protein gel is strongly dependent on the balance between attraction and repulsion forces among denatured protein molecules during aggregation. With decreasing intermolecular repulsion obtained by increasing the ionic strength, the gel networks become coarser, being composed of more particulate aggregates, which can be as large as micrometers.

Nevertheless, the effect of NaCl concentration was small. This could be explained by the $CaCl_2$ concentration of the powder and therefore in the gel. After hydration, the $CaCl_2$ concentration was 0.162 and 0.179 g/100 g for 29.13 and 32.17 g/100 g protein concentrations, respectively.

Barbut et al. (*5, 30*) and Bryant et al. (*10*) demonstrated the high sensitivity of the gel microstructure and rheological properties increasing in $CaCl_2$ as compared to NaCl and concluded that at high $CaCl_2$ concentrations the strength is relatively insensitive to salt concentration.

Verheul et al. (*4*) measured permeability of whey protein gels (3.5 and 6.2 g/100 g) as a function of NaCl concentration. An increase in permeability was found with increasing NaCl concentrations, indicating an increase in pore size, and confirmed by SEM pictures of such gels. Moreover, they observed a smaller increase in permeability with an increase in whey protein concentration (from 3.5 to 6.2 g/100 g) and they concluded that the pores in the gel were smaller when more proteins were present. This was in accordance with the SEM images of this study and the variations in D_{PEG}. A decrease in pore size in relation to C_{NaCl} was observed in our study due to the high whey protein concentrations used as compared to those of Verheul et al. (*4*).

All of these findings are in accordance with the diffusion data. As seen on SEM images, PEG diffusion decreased when

5112 *J. Agric. Food Chem.,* Vol. 54, No. 14, 2006

Colsenet et al.

C_{NaCl} increased because the NaCl considerably reduced the distances between the aggregates and the porous structure of the gel.

Measurement of PEG self-diffusion in whey protein gels demonstrated changes in gel structure according to treatment conditions. Two opposite effects were observed, i.e., a decrease in PEG diffusion when salt concentrations increased and an increase in PEG diffusion with increasing denaturation time.

Increasing the heating time contributed to thickening of the gel network by incorporation of more denatured proteins. A decrease in the number of native proteins in the aqueous phase thus involves a decrease in aqueous phase viscosity. At the same time, a network with large spaces was created as seen on SEM images. The increase in diffusion with denaturation was dependent on the probe size. The observation of PEG and protein diffusion reflected the protein denaturation and was the consequence of PEG/protein friction. When the diffusion became constant, the gel point was reached and all of the whey proteins were denatured.

The addition of NaCl to whey protein solution contributed to densification of the gel by screening the repulsion forces between the proteins. The SEM images showed that the gels formed with high NaCl concentrations were very dense, reflecting a higher degree of branching and cross-linking. The densification of the network involved reduction of D_{PEG} in gels.

ACKNOWLEDGMENT

LITERATURE CITED

(1) Kilara, A.; Vaghela, M. N. *Dans Proteins in Food Processing*; Woodhead Publishing in Food Science and Technology: Cambridge, 2004.

(2) Renard, D. Etude de l'agrégation et de la gélification des protéines globulaires: Application à la β-lactoglobuline. Thesis, Université de Nantes, 1994.

(3) Verheul, M.; Roefs, S. Structure of particulate whey protein gels: Effect of NaCl concentration, pH, heating temperature, and protein composition. *J. Agric. Food Chem.* **1998**, *46*, 4909–4916.

(4) Verheul, M.; Roefs, S. Structure of whey protein gels, studied by permeability, scanning electron microscopy and rheology. *Food Hydrocolloids* **1998**, *12*, 17–24.

(5) Barbut, S. Effect of sodium level on the microstructure and texture of whey protein isolate gels. *Food Res. Int.* **1995**, *28*, 437–443.

(6) Roefs, S.; Dekruif, K. G. A model for the denaturation and aggregation of β-lactoglobulin. *Eur. J. Biochem.* **1994**, *226*, 883–889.

(7) Kinekawa, Y.; Fuyuki, T.; Kitabatake, N. Effects of salts on the properties of sols and gels prepared from whey protein isolate and process whey protein. *J. Dairy Sci.* **1998**, *81*, 1532.

(8) Stading, M.; Langton, M.; Hermansson, A. M. Microstructure and rheological behaviour of particulate β-lactoglobulin gels. *Food Hydrocolloids* **1993**, *7*, 195–212.

(9) Caussin, F.; Famelart, M. H.; Maubois, J. L.; Bouhallab, S. Mineral modulation of thermal aggregation and gelation of whey proteins: From β-lactoglobulin model system to whey protein isolate. *Lait* **2003**, *83*, 1–12.

(10) Bryant, C.; McClements, D. J. Influence of NaCl and CaCl2 on cold-set gelation of heat-denatured whey protein. *J. Food Sci.* **2000**, *65*, 801–804.

(11) Le Bon, C.; Nicolai, T.; Kuil, M. E.; Hollander, J. G. Self-diffusion and cooperative diffusion of globular proteins in solution. *J. Phys. Chem. B* **1999**, *103*, 10294–10299.

(12) Beretta, S.; Chirico, G.; Baldini, G. Short-range interactions of globular proteins at high ionic strengths. *Macromolecules* **2000**, *33*, 8663–8670.

(13) Croguennoc, P.; Nicolai, T.; Kuil, M. E.; Hollander, J. G. Self-diffusion of native proteins and dextran in heat-set globular protein gels. *J. Phys. Chem. B* **2001**, *105*, 5782–5788.

(14) Colsenet, R.; Soderman, O.; Mariette, F. Pulsed field gradient NMR study of Poly(ethylene glycol) diffusion in whey protein solutions and gels. *Macromolecules* **2006**, *39* (3), 1053–1059.

(15) Stejskal, E. O.; Tanner, J. E. Spin diffusion measurements: Spin–echoes in the presence of time-dependent Field Gradient. *J. Chem. Phys.* **1965**, *42*, 288–292.

(16) Tanner, J. E. Use of stimulated echo in NMR diffusion studies. *J. Chem. Phys.* **1970**, *52* (5), 2523–2526.

(17) Ikeda, S.; Morris, V. J. Fine-stranded and particulate aggregates of heat-denatured whey proteins visualized by atomic force microscopy. *Biomacromolecules* **2002**, *3*, 382–389.

(18) Aymard, P.; Durand, D.; Nicolai, T. The effect of temperature and ionic strength on the dimerisation of β-lactoglobulin. *Int. J. Biol. Macromol.* **1996**, *19*, 213–221.

(19) Colsenet, R.; Söderman, O.; Mariette, F. Diffusion of poly-(ethylene glycol)s in casein suspensions and gels: A pulsed field gradients NMR study. *Macromolecules* **2005**, *38*, 9171–9179.

(20) Koppel, D. E. Analysis of macromolecular polydispersity in intensity correlation spectroscopy–Method of cumulants. *J. Chem. Phys.* **1972**, *57*, 4814.

(21) Callaghan, P. T.; Pinder, D. N. A pulsed field gradient NMR study of self-diffusion in a polydisperse polymer system–Dextran in water. *Macromolecules* **1983**, *16*, 968–973.

(22) Alper, J. S.; Gelb, R. I. Standard errors and confidence intervals in nonlinear regression: Comparison of Monte Carlo and parametric statistic. *J. Phys. Chem.* **1990**, *94*, 4747–4751.

(23) De Gennes, P. G. Brownian motions of flexible poymer chains. *Nature* **1979**, *282*, 367–370.

(24) Tao, H.; Lodge, T. P.; von Meerwall, E. D. Diffusivity and viscosity of concentrated hydrogenated polybutadiene solutions. *Macromolecules* **2000**, *33*, 1747–1758.

(25) Zhang, G. Y.; Foegeding, E. A. Heat-induced phase behavior of β-lactoglobulin/polysaccharide mixtures. *Food Hydrocolloids* **2003**, *17*, 785–792.

(26) Clark, A. H.; Kavanagh, G. M.; Ross-Murphy, S. B. Globular protein gelation–Theory and experiment. *Food Hydrocolloids* **2001**, *15*, 383–400.

(27) Zhu, R. H.; Frankema, W.; Huo, Y. L.; Kok, W. T. Studying protein aggregation by programmed flow field-flow fractionation using ceramic hollow fibers. *Anal. Chem.* **2005**, *77*, 4581–4586.

(28) Ikeda, S. Heat-induced gelation of whey proteins observed by rheology, atomic force microscopy, and Raman scattering spectroscopy. *Food Hydrocolloids* **2003**, *17*, 399–406.

(29) Langton, M.; Hermansson, A. M. Fine-stranded and particulate gels of beta-lactoglobulin and whey-protein at barying pH. *Food Hydrocolloids* **1992**, *5*, 523–539.

(30) Barbut, S. Effects of calcium level on the structure of preheated whey protein isolate gels. *Food Sci. Technol. Lebensm.-Wiss. Technol.* **1995**, *28*, 598–603.