Power Law Behavior of Structural Properties of Protein Gels

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Whey proteins are globular, heat-sensitive proteins. The gel structure, the formation of this structure, and the rheological properties of particulate whey protein isolate (WPI) gels have been investigated. On increasing the NaCl concentration, the permeability of the WPI gels increased, indicating a coarsening of the gel structure, confirmed by confocal scanning laser microscopy pictures. Only a part of the total amount of protein present contributed to the gel network at the gel point (the primary spatial structure). Large variations were observed in the amount of aggregated material at the gel point (and thus the primary spatial structure) as a function of NaCl concentration, due to differences in the kinetics of the denaturation/aggregation process. After the gel point more protein is incorporated in the gel network by "thickening" the strands in the gel and "decorating" the pores in the gel, apparently without changing the gross spatial structure. Power law behavior was found for the permeability dependence of aged gels on the amount of aggregated material *at* the gel point. For various salt concentrations the curves coincided to one master curve. This power law behavior is consistent with a primary spatial structure of fractal flocs with a fractal dimensionality of 2.4. The elastic modulus is remarkably related (via a power law) with the *total* amount of protein contributing to the gel network, in contrast to permeability.

Introduction

An important phenomena for (bio)polymers is gelation, which has been subject to a number of theoretical studies.[1−3] The gelation of biopolymers is induced by several forces such as chemical bonding, electrostatic interactions, hydrogen bonding, van der Waals forces, and hydrophobic interactions.[4,5] Consequently, it is rather difficult to describe the gelation mechanism of biopolymers, which is of great importance for the structural and macroscopic viscoelastic properties of the resultant gels. In particular there is a large variation in structural properties of biopolymer gels.[6,7]

In this paper we focus on heat-induced whey protein gels. Whey is the residual fluid after cheese making. The main whey proteins in bovine whey are β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumin (BSA), and immunoglobulins (Ig's), with β-lg constituting more than 50% of the total whey protein.[5] These whey proteins are mainly globular and heat-sensitive. Upon heating, they undergo conformational changes (denaturation) and subsequently aggregate irreversibly via noncovalent and covalent bonds; eventually they will form a gel.[8,9]

Heat-induced globular protein aggregates and gels have previously been studied by several experimental techniques, such as rheology,[10−12] microscopy,[13−16] light scattering,[17−19] small-angle neutron scattering,[17,20,21] water-holding capacity,[22,23] and gel permeability.[19,24−26] Struc-

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tural and physical properties of heat-induced globular protein gels can vary widely and are dependent upon heating conditions, protein concentration, pH, ionic strength, and type of ions.[13,19,27−29] Transparent gels with fine-stranded structures are formed under conditions of large electrostatic repulsion between the proteins, that is, at low ionic strength and far from the isoelectric point of the proteins. At high ionic strength and close to the isoelectric point of the proteins, turbid, milk-white gels with particulate gel structures are formed.[10,13,14,17,29,30]

The structure of particulate (heat-induced) globular protein aggregates and gels has been described using the concept of fractals.[10,16−21,24,25] The number of particles in a fractal floc depends on the radius of this floc (R_{floc}) via a power law; the exponent of the power law is called the fractal dimension D. During the aggregation process the fractal clusters grow until they occupy the total liquid volume, at which moment a gel is formed. This implies that the size of the flocs in the gel is determined by the volume fraction of particles building up the gel (ϕ) and the fractal dimensionality of the flocs:[19]

$$R_{floc} = a\phi^{1/(D-3)} \quad (1)$$

in which a is the radius of the building particle in the fractal flocs. Scaling relations have been derived between macroscopic properties (elastic modulus and gel permeability) and the volume fraction of building particles of a fractal gel.[16,19,31,32]

In a previous study we noticed that for whey protein gels, structural properties, such as dynamic moduli and gel permeability, strongly depend on the NaCl concentration in the medium.[24] Permeability mainly depends on the large pores in the gel network, and permeability measurements are very useful for probing gel structure on a micrometer length scale. Around neutral pH, gels of whey protein isolate (WPI) made at an ionic strength of 0.1−0.2 M or higher can be identified as particulate,[24,27] and gel rigidity decreases, while permeability increases enormously with increasing ionic strength after a given heating time.[22,24,33] This is in agreement with the observed decrease in water-holding capacity of this kind of system.[22] In our previous work we also revealed the important fact that only a part of the total protein present is aggregated at the time a gel is formed.[24] In this paper we will show that the large-scale gel structure does not change much after the gel point. Moreover, combining aggregation kinetics with the development of dynamic moduli and permeability during aging enables us to scale these structural properties for gels made under different conditions. The results are discussed in view of the fractal concept. This study is a step forward in the understanding of the gelation of globular proteins, as it clearly relates gelation and structural and viscoelastic properties.

Materials and Methods

Preparation of WPI Dispersions and Gels. A commercial whey protein isolate (WPI) powder (Trade name Bipro, produced by Davisco International Inc. (USA) and purchased from Domo Food Ingredients, Beilen (The Netherlands)) was used for the experiments. The powder contained approximately 89% (w/w) protein (70% (w/w) β-lg, 11% (w/w) α-la, 5% (w/w) Ig, 4% (w/w) BSA), 2% (w/w) ash, less than 1% (w/w) lactose and 4% (w/w) water. WPI dispersions (total protein concentrations of 27−135 g L^{-1}) were prepared by dissolving WPI powder in 0.1−3.0 M NaCl solutions that were made using double-distilled water. For the experiments in the previous study, WPI dispersions were used for which the pH was not adjusted, and so the pH shifted to lower values (from pH 7.1 to pH 6.8) with increasing NaCl concentration (from 0.1 to 3.0 M).[24] To be sure that the effects measured were only due to the effect of the salt, in the present study the pH of the dispersions was, unless otherwise stated, adjusted to 6.8 using HCl or NaOH. The WPI dispersions were stirred for at least 2 h, centrifuged for 10 min at 20000*g*, and filtered using a 0.45-μm non-protein-adsorbing filter to remove insoluble materials. The WPI concentrations given in the Results and Discussion are total protein concentrations. WPI gels were made by heating the WPI dispersions at 68.5 °C.

Conversion of Native Whey Proteins. WPI dispersions were heated in test tubes at 68.5 °C for different time periods. The tubes were cooled in ice water, the pH was adjusted to 4.7 ± 0.1, and the aggregated proteins were sedimented by centrifugation for 30 min at 20000*g*. The concentrations of the residual native whey proteins in the supernatant were determined by high-performance gel permeation chromatography (HP-GPC).[34]

Visual Determination of Gel Point and Conversion of Native Whey Protein at Gel Point. WPI dispersions were heated in test tubes at 68.5 °C for different time periods. The tubes were cooled in ice water for 5 min and stored at room temperature for another 55 min. Next, the tubes were turned gently upside down. The WPI dispersion in the tube that did not fall down on turning the tube was considered to be a gel, and the shortest heating time at which a gel formed was defined as the gel point. The concentrations of residual native protein were determined by HP-GPC as described above. In the Results and Discussion errors in the aggregated concentrations are represented as a mean deviation, which was calculated from the values measured at the gel point and in the tubes just before and just after the gel point.

As well as the visually determined gel point, the time that G', the elastic modulus, started to deviate significantly from zero was used as the gel point.

Permeability Measurements. The permeability coefficient, B_{gel}, was determined from the liquid flux of a NaCl solution through glass tubes filled with a WPI gel, due to a hydrostatic pressure gradient. B_{gel} was measured at 20 °C after cooling the tubes with gels in ice water. The method has been extensively described before[24,25] and is based on the method developed by van Dijk and Walstra.[26] B_{gel} values in the Results and Discussion are mean values taken from 12 readings, and errors are represented as the standard deviation of the mean value.

Rheological Measurements. Rheological measurements were made with a Rheometrics RFS II rheometer. A couette geometry was used (cup diameter, 17 mm; bob diameter, 16.5 mm; bob length, 12.9 mm). A WPI dispersion of 2 mL was heated at 68.5 °C in the measuring cup of the rheometer, which was preheated at this temperature. The sample was covered with a layer of hexadecane oil to prevent evaporation. During the gelation process an oscillating strain was applied to the sample with a frequency of 1 rad s^{-1} and a maximum strain of 1%. This was well within the linear viscoelastic region. The resulting time-dependent stress was recorded and used to calculate G', the storage modulus, and G'', the loss modulus.

Confocal Scanning Laser Microscopy. WPI dispersions were prepared as described above; the protein was stained before heating with Rhodamine B (approximately 0.25 mg per gram of protein), which is a fluorescent dye. Dispersions were put on special glass slides in which holes of 0.5 mm were polished and were covered with glass cover slips. The glass slides were put on a glass dish, in which some oil was put to improve heat transfer. The glass dish with the slides was put in a water bath and heated

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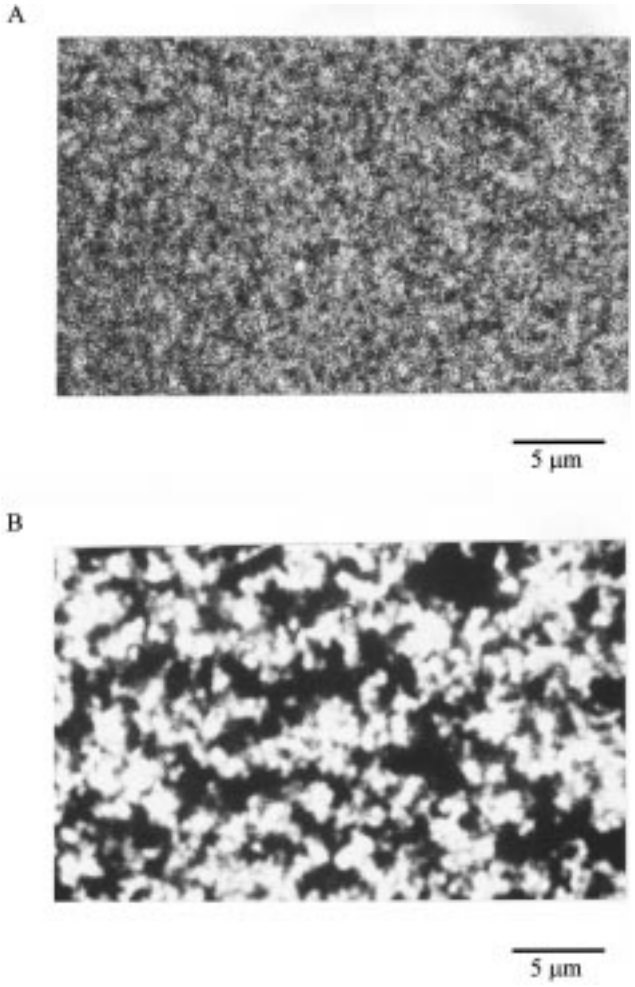

Figure 1. CSLM pictures of 89 g L^{-1} WPI heated for 20 h at 68.5 °C: (A) 0.1 M NaCl, pH 7.1; (B) 0.5 M NaCl, pH 6.9.

for 20 h at 68.5 °C. After the dish was cooled to room temperature, the WPI gels were studied by confocal scanning laser microscopy (CSLM). CSLM is a technique in which the sample is scanned by a focused laser beam.[16,35] The technique has the advantage that the fluorescent sample can be studied in the hydrated state, without further fixation and dehydration procedures which may affect the structure. Because of the limited resolution (>0.2 μm), the technique is suited to studying structures at longer (μm) length scales. In this study the CSLM (BioRad MRC 500) facility at CDI-DLO in Lelystad (The Netherlands) was used. Pictures were taken at a depth of approximately 10 μm from the glass surface in order to minimize surface effects.

Results and Discussion

CSLM. In Figure 1 we show CSLM pictures of WPI gels in 0.1 M NaCl and in 0.5 M NaCl after heating for 20 h at 68.5 °C. They show that the structure of the gel is much coarser at higher salt concentrations. Gels made at NaCl concentrations between 0.1 and 0.5 M showed intermediate structures. If examined at different length scales, the gels may appear similar, which indicates that they may have a fractal-like structure.[16,19] Because of the limited resolution of the CSLM pictures, it was not possible to determine experimentally the density correlation function and see whether the structure is fractal over a certain length scale.[16]

Aging Time. An extremely important point, which is usually not addressed in the literature, is the relationship between structural properties and conversion of native

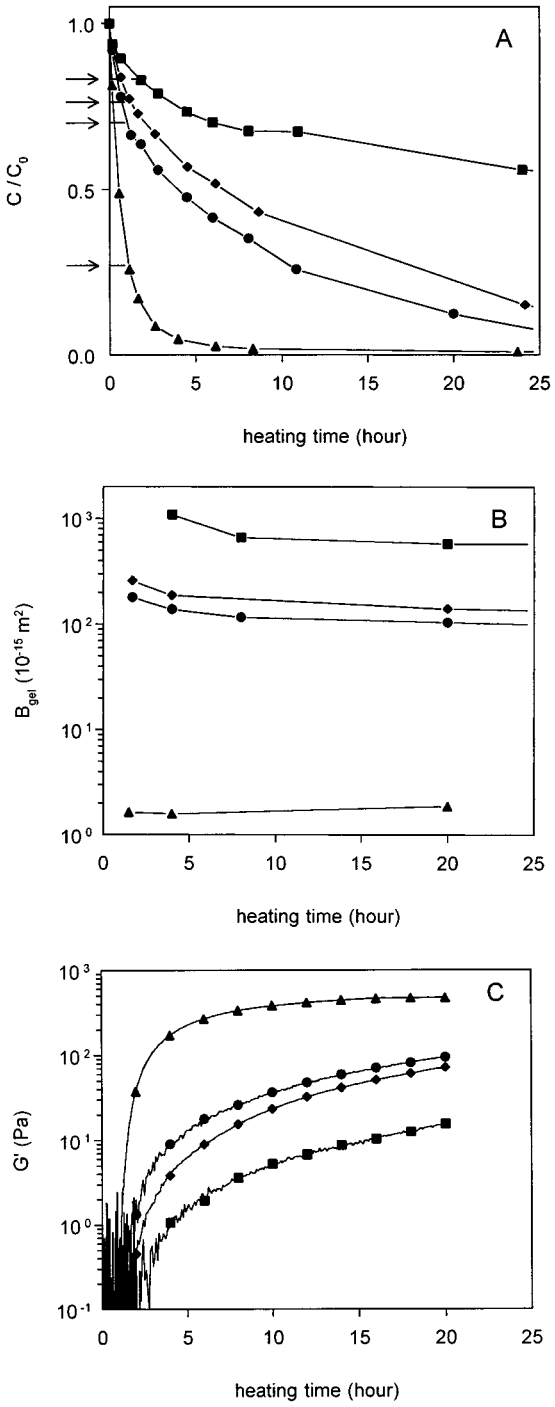

Figure 2. Fraction of nonaggregated protein (A), B_{gel} (B), and G' (C) as a function of heating time at 68.5 °C for 44.5 g L^{-1} WPI: (▲) 0.1 M NaCl, pH 7.1; (●) 0.5 M NaCl, pH 6.8; (◆) 0.7 M NaCl, pH 6.9; (■) 3.0 M NaCl, pH 6.8; the arrows indicate the gel points.

protein. Often, it is not recognized that at the gel point not all protein present makes up the gel network or that the amount constituting the network increases after the gel point. This is illustrated by Figure 2. The rate at which the fraction of native protein decreases in time strongly decreases with NaCl concentration (Figure 2A) as well as the fraction of protein aggregated at the gel point. The permeability coefficient, B_{gel}, which was determined at four different NaCl concentrations (Figure 2B), strongly increased with salt concentration. Although the curves are shifted over 3 orders of magnitude, they have a similar shape. B_{gel} decreases by at most a factor

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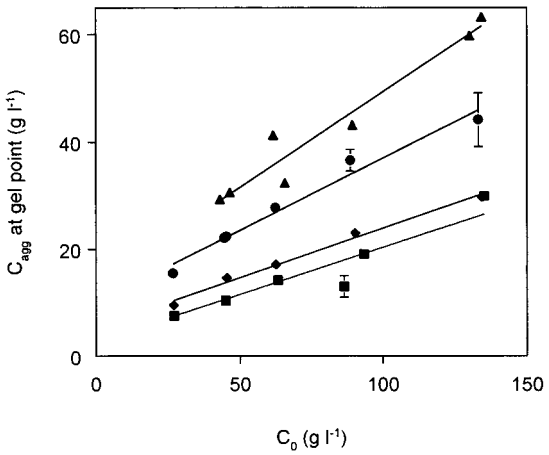

Figure 3. Concentration of aggregated protein at the gel point versus initial protein concentration for WPI at pH 6.8 heated at 68.5 °C. NaCl concentrations: (▲) 0.1 M; (●) 0.2 M; (◆) 0.5 M; (■) 3.0 M.

of 2 upon further heating after the gel point, which is appreciable in itself but small compared to the differences between the gels. Thus, the geometry of the gel network (the primary spatial structure) is mainly established at the gel point. The elastic modulus, G', on the other hand, which mainly probes the amount of material in the gel network and the interaction between the structural units, increases strongly after the gel point and levels off when all the protein has aggregated (Figure 2C).

Scaling Behavior of Gel Permeability. At the gel point, fractal-like gel structures are formed (Figure 1), and the amount of aggregated protein (C_{agg}) at the gel point is responsible for building this structure. Figure 3 shows C_{agg} at the gel point as a function of the initial protein concentration, C_0 (the gel point was determined by visual observation). At low NaCl concentrations much more protein is transformed into aggregates than at high NaCl concentrations. This is reflected in the much lower B_{gel} value found at low NaCl concentration and a fractal character of the gels, since more material leads to smaller fractal flocs and thus, a finer, less permeable gel network.

The large-scale geometry of whey protein gels made at different salt concentrations only depends on the amount of protein aggregated at the gel point. This is shown in Figure 4 for WPI gels made in a range of protein and salt concentrations. By plotting B_{gel} against C_{agg} at the gel point (Figure 4B), instead of against C_0 (Figure 4A), the experimental points coincide to a master curve for 0.2−1.0 M NaCl. The NaCl concentration and protein concentration determine the time of gelation and the amount of protein aggregated at the gel point. At 0.1 M NaCl, B_{gel} values are slightly lower than the master curve. This salt concentration, which coincides with a change in the kinetics of the aggregation process, is also at the transition from fine-stranded to particulate gel structures.[10,36] The scaled curve in Figure 4B is analyzed using the fractal scaling relations derived by Bremer et al.[19,37] The fractal gel is regarded as consisting of fractal flocs, which are approximated as spherical blobs. From the Kozeny−Carman equation,[38] which is valid for dense and isotropic

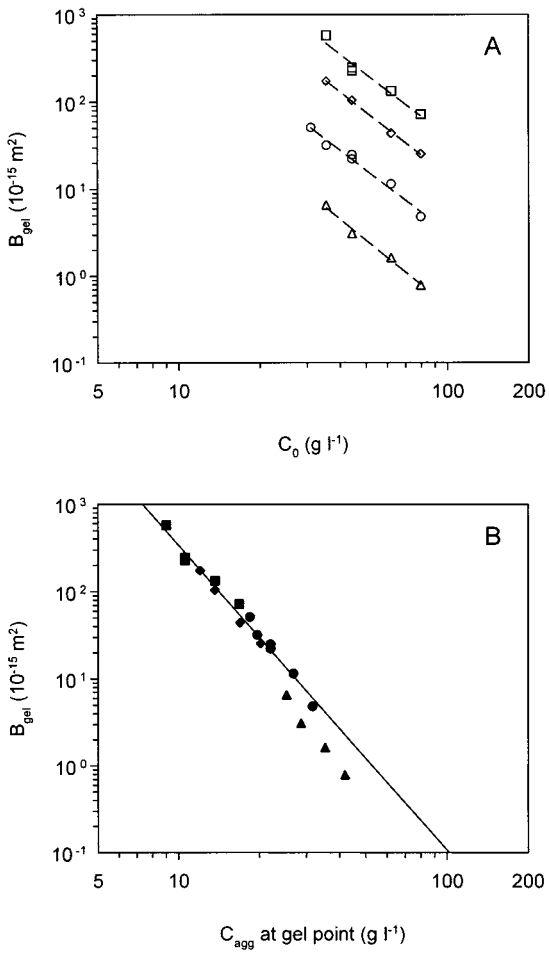

Figure 4. B_{gel} after heating at 68.5 °C for 20 h versus (A) C_0 and (B) C_{agg} at the gel point for various initial WPI concentrations at pH 6.8. NaCl concentrations: (▲, △) 0.1 M; (●, ○) 0.2 M; (◆, ◇) 0.5 M; (■, □) 3.0 M.

sphere packings,[39] the liquid permeability of a fractal gel can be derived:[19,37]

$$B_{gel} = KR_{floc}^{\ 2} = Ka^2\phi^{2/(D-3)} \qquad (2)$$

K is a constant, comparable with the reciprocal of the tortuosity factor in the Kozeny−Carman equation,[40] and is dependent on the cluster size distribution and on the ratio between the effective hydrodynamic radius and the radius of the flocs in the gel. Although the protein concentration in the whey protein gels is rather low, the packing of the blobs in the gels can be considered as dense, since the fractal flocs pack into space-filling networks. For the whey protein gels, ϕ is proportional to C_{agg} at the gel point. The fractal dimensionality of the flocs that build up the WPI gels, calculated from eq 2 and Figure 4B, is $D \approx 2.4$.

R_{floc} was assessed from eq 2 and experimental B_{gel} values after heating for 20 h for initial WPI concentrations of 44.5 and 89.0 g L^{-1}. For this, the constant K in eq 2 was assumed to be 0.01, as was found experimentally for polystyrene latex gels and had led to a consistent interpretation of other particulate protein (casein) gels with B_{gel} values in the same range.[37] Table 1 shows that floc size increases strongly with NaCl concentration and

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Table 1. R_{Floc} Calculated from Experimental B_{Gel} Values, Using Eq 2 and $K = 0.01$

	R_{floc} (μm)	
[NaCl] (M)	$C_0 = 44.5$ g L^{-1}	$C_0 = 89$ g L^{-1}
0.1	0.56	0.25
0.2	1.58	0.66
0.5	3.23	1.39
3.0	4.77	2.32

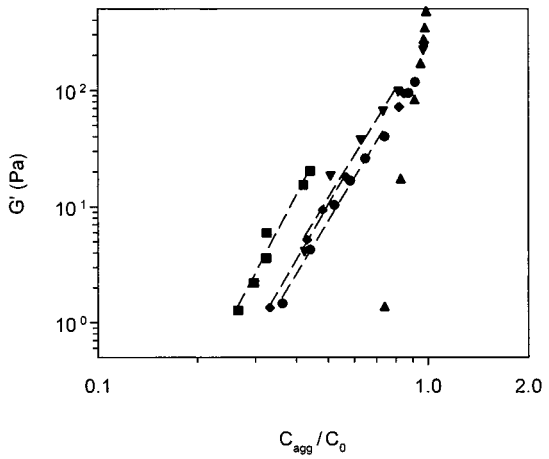

Figure 5. G' versus fraction of aggregated protein during heating 44.5 g L^{-1} WPI at 68.5 °C: (▲) 0.1 M NaCl, pH 7.1; (▼) 0.4 M NaCl, pH 7.0; (●) 0.5 M NaCl, pH 6.8; (◆) 0.7 M NaCl, pH 6.9; (■) 3.0 M, pH 6.8.

decreases with protein concentration. The global sizes of the flocs and the differences in size between 0.1 and 0.5 M NaCl (factor 5−6) are found in the CSLM pictures of Figure 1.

Power Law Behavior of Elastic Modulus. The elastic modulus, G', starts to increase after the gel point, when the primary spatial structure is established. The increase in G' is coupled with an additional aggregation of protein. In Figure 5, G' is plotted versus the total amount of aggregated protein for five different gels. The curves are very similar and close together, except for the curve of 0.1 M NaCl. As mentioned above, this salt concentration coincides with a transition in aggregation kinetics and it marks the transition in gel structure from fine-stranded to particulate.[10,36] Further, the differences between the 0.4 and 3.0 M salt measurements are significant. Therefore, we drew some lines to guide the eye. The results confirm that the elastic modulus mainly probes the total amount of protein in the gel. The spatial structure has only a minor influence. Differences between the curves may be further due to the lower amounts of protein aggregated at the gel point and stronger interparticle forces with increasing salt concentration. The latter may be a consequence of deeper attractive potentials between molecules and/or particles. Curiously enough, the gel modulus scales with the amount of protein making up the network at a given NaCl concentration; the scaling exponent is 5 ± 0.5 for 0.4−3.0 M NaCl. This value is close to values found for fractal networks before,[31,32] although after the gel point the protein gel network is no longer the result of a fractal aggregation process (see also Figure 6)!

Structure of Whey Protein Gels. We have shown before that WPI dispersions form particulate gels at neutral pH and NaCl concentrations ≥ 0.1 M and a heating temperature of 68.5 °C.[24] These gels were described as nested structures: native protein molecules form building particles of around 0.1 μm, which in turn form a space-filling gel structure, in accordance with the structure of β-lactoglobulin aggregates as described by Aymard et al.[21] Summarizing the results from CSLM, gel permeability, aggregation kinetics, and dynamic rheology from the present study, we think that the gels formed can be considered as self-similar, fractal networks at the point of formation (Figure 6A). At the gel point only part of the protein present makes up the building of this fractal network (the primary spatial structure) and determines the size of the flocs in the gel. Prolonged heating does not change the nature or large-scale structure of the primary spatial structure. The network is "thickened" and "strengthened" or "decorated" by the rest of the protein (Figure 6B), and permeability decreases by a factor of 2 only. Apparently, the strengthening of the strands and links does not change the gross spatial structure. This is remarkable in comparison to transient gels, in which reorganization and growth of the clusters leads to the "collapse" of the network.[41,42] Figure 6 gives a schematic representation of the fractal structure at the gel point (Figure 6A) and the gel structure after prolonged heating (Figure 6B). It shows the "decoration" of the pores in the gel, which gives a (small) decrease in pore size (and thus a decrease in B_{gel}) and an increase in the amount of strands between the flocs. The strands in the flocs may be "thickened" and thus strengthened as well. The second process, in which the additional part of the protein above the gel point is incorporated in the network, is different from the first one, that is, the (fractal) aggregation and gelation in itself.

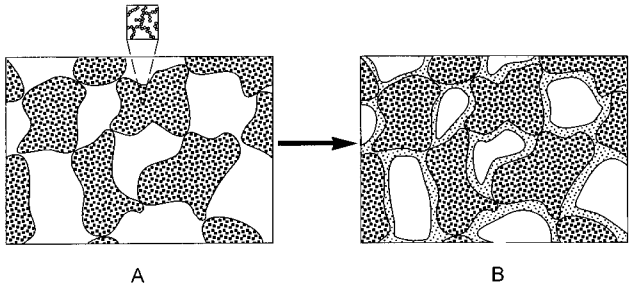

Figure 6. Schematic representation of the fractal gel structure at the gel point (A) and the "decoration" of the pores in the gel structure after prolonged heating (B).

Conclusions

At pH 6.8 whey protein dispersions form particulate gels by heat treatment at 68.5 °C and NaCl concentrations above 0.1 M NaCl. Combination of measurements on gel permeability, rheology, and aggregation kinetics as a function of heating time has led to a clearer picture of the heat-induced gelation process of whey proteins. In the initial stage of the gelation process a primary spatial structure is formed by only part of the total amount of protein present. In the next stage, the structure is only affected by a thickening (and strengthening) of the strands that build the structure and a decoration of the pores through incorporation of the rest of the protein in the network. The large increase in permeability with NaCl concentration as found for the WPI gels was explained solely by the large decrease in the amount of protein constituting the primary spatial structure, and scaling behavior was found between B_{gel} and the concentration of aggregated protein at the gel point. Gel rigidity appeared

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to be related via a power law with the total amount of protein that is incorporated in the gel structure at all heating times. The primary spatial structure could be well-described as consisting of fractal flocs with a fractal dimensionality of 2.4.

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