Articles

Rheological Study on the Fractal Nature of the Protein Gel Structure

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The effects of ionic strength on fractal structures in heat-induced gels prepared from globular proteins were investigated in the framework of a fractal aggregation of colloidal particles. All gels formed at 90 °C exhibited power law relationships between the storage shear modulus (G') and protein concentration. At 25 mM NaCl, the fractal dimension, d ($\sim$2.2), calculated based on the value of the power law exponent agreed with those for reaction-limited cluster−cluster aggregation. Further addition of NaCl (50, 80, 500, 1000 mM) decreased the values of d ($\sim$1.8), which agreed with d for diffusion-limited cluster−cluster aggregation. These results suggest that the predominant effect of an increase in ionic strength on globular protein gelation is ascribed to shielding charges on the surface of the proteins, thereby increasing the reaction probability of protein aggregation. The effective structure-determining rheological properties of heat-induced protein gels are characterized by fractal dimensions deduced from the rheological model, which also suggests that the size of primary flocs building fractal structures is one of the important factors that determines the linear viscoelastic properties of the gels.

Introduction

The aggregation of globular proteins is regulated by many factors such as covalent bonding, electrostatic interactions, hydrogen bonding, hydrophobic interactions, and van der Waals forces.[1−3] If the external conditions are changed, the protein in solution may unfold and expose interior hydrophobic regions and sulfydryl groups to the solvent.[4−6] The partially denatured proteins can aggregate and, under appropriate conditions, produce a macroscopically continuous three-dimensional gel network that entraps and restricts the motion of the solvent.[3] While the gelation phenomenon of globular proteins has generated much interest not only from practical necessity but from the scientific point of view, the mechanisms creating network structures of protein gels are yet unclear.

Recently, various experimental techniques in rheology, microscopy, scattering, and gel permeability have been applied to elucidate fractal structures in aggregates or gels of proteins such as β-lactoglobulin, bovine serum albumin (BSA), caseins, and soy proteins.[7−15] In the framework of fractal theories, the network structure of protein gels is considered to be a collection of fractal aggregates closely packed throughout the system. The number of particles inside a fractal aggregate, N_p, depends on the size of the aggregates, ξ, through a power law relationships: $N_p \sim (\xi/a)^d$, where a is the size of the primary particles and the exponent is the fractal dimension, d. The physical properties of the aggregates can be related to the structural properties through scaling relationships predicted based on fractal concepts,[7−9,16−18] and the fractal dimension is evaluated using experimentally obtained scaling exponents. Hagiwara et al. have shown based on microstructural images that globular proteins such as β-lactoglobulin, BSA, and soy proteins formed heat-induced gels with fractal structures in the scale length range of ca. 0.1−10 μm.[13,14] In addition, the values of the fractal dimension evaluated from the images are consistent with those evaluated using rheological data and a model developed by Shih et al.[17] Vreeker et al. also investigated heat-induced β-lactoglobulin gels and showed that the

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rheological model by Shih et al.[17] gave values of the fractal dimension consistent with those obtained by a static light scattering method.[10] While scattering techniques are considered to be one of the most powerful methods to analyze fractal structures of colloidal aggregates, experimental conditions such as turbidity or concentration of the samples or applicable scale length are rather limited. Microscopic image analyses are the most direct method for evaluating fractal dimension. However, it is quite difficult to obtain an image of an intact gel due to damages during sample preparation. In contrast, rheological measurements are easy to conduct, especially in the high-concentration range of proteins. The rheological fractal model by Shih et al.[17] is therefore expected to provide a tool for understanding the relationship between the structural and rheological properties of globular protein gels.

The ionic strength of a monovalent salt is known to vary the microscopic network structure of globular protein gels.[2,3,19−21] At a low ionic strength, the proteins show a tendency toward forming linear aggregates, with a low degree of branching, leading to a transparent network composed of fine strands with a diameter of several nanometers (the so-called fine-stranded network),[1] while at a high ionic strength, a random aggregation occurs, leading to the formation of an opaque particulate network in which the strands have dimensions in the micrometers (the so-called particulate network).[21] Therefore, the microstructures of gel networks transform from fine-stranded to a mixture of fine strands and spherical aggregates and then to particulate networks when the ionic concentration of a monovalent salt is increased from 25 to 500 mM.[22−26] Since these changes are not associated with changes in the secondary structure of the protein,[2] adding a monovalent salt seems to change the aggregation conditions by altering the balance between attractive and repulsive forces through electrostatic shielding effects.

In this study, we applied the rheological fractal model developed by Shih et al.[17] to the properties of heat-induced whey protein isolate (WPI) gels. WPI is a practically important source of globular milk proteins, consisting mainly of β-lactoglobulin, α-lactalbumin, BSA, and immunoglobulins, where β-lactoglobulin dominates the gelation properties.[3] We focus on how the ionic strength of a monovalent salt alters the heat-induced gelation of the globular proteins. Dynamic small-strain rheometry and large-strain torsional testing were used to determine the rheological properties of gels prepared from protein solutions containing 25−1000 mM NaCl.

Theoretical Considerations

Scaling Behavior of Gel Elasticity. In the theory of Shih et al.,[17] based on the work of Brown,[27] Buscall et al.,[28] and Kantor and Webman,[29] the gel network is considered as closely packed fractal flocs with the fractal dimension of d. The elastic properties of a floc are dominated by its effective backbone, which can be approximated as a linear chain of springs. The elastic constant (K) of the individual flocs is inversely related to their size (ξ):

$$K \propto K_0/\xi^{2+x} \qquad (1)$$

where K_0 is the local bending constant between two adjacent springs that belong to the effective backbone of a floc and x is the fractal dimension of the elastic backbone. Since fractal flocs are considered scale invariant, the size of the flocs is related to the volume fraction (ϕ) as $\xi \propto \phi^{1/(d-3)}$.

In the case where the links between the neighboring flocs have a higher elasticity than those in the flocs, which is the so-called strong-link regime, the macroscopic elastic constant of the gel (G) is dominated by K. Since G of a system of size L can be related to K as $G \propto (L/\xi)K$, the dependence of G on ϕ is derived:

$$G \propto \phi^{(3+x)/(3-d)} \qquad (2)$$

When strain imposed on the system is increased, the breaking of the weakest bond occurs in the flocs. The limit of linearity (γ_0) at which the weakest bond breaks decreases with increasing ϕ in the strong-link regime because the flocs become more brittle with decreasing size (a fixed magnitude of strain leads to larger force) (eq 1).[17]

If the size of the flocs is sufficiently small, the flocs are more rigid than the interfloc links and, thus, the elasticity of the gel is dominated by that of the interfloc links. The breaking of bonds with increasing strain occurs at a interfloc link. In this weak-link regime, γ_0 is expected to increase with increasing ϕ because the deformation of a interfloc link becomes less with increasing ϕ.[17]

Cluster−Cluster Aggregation. The process of colloidal aggregate formation has also been successfully investigated based on fractal considerations.[30] Fractal growth models have been applied to the aggregation process of particles.[30] In the cluster−cluster aggregation process, diffusing particles in a certain medium stick to one another at contact in a random way with probability p. The resultant aggregate or cluster diffuses and forms larger clusters by linking to other clusters or particles. Two limiting regimes of cluster−cluster aggregation have been investigated: reaction-limited and diffusion-limited cluster−cluster aggregation, corresponding to the situation where $p \ll 1$ and $p \sim 1$, respectively. In the case of reaction-limited aggregation, aggregates are more coase and open-space than those formed in the diffusion-limited process and linear strand-like structure can be locally seen. The results of computer simulations and experiments have revealed that aggregates grown in the reaction-limited and diffusion-limited aggregation regimes are characterized by fractal dimensions of 2.0−2.2 and 1.7−1.8, respectively.[10−12,31−33]

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Materials and Methods

Materials. A commercial whey protein isolate (WPI) (Davisco Foods International, LeSueur, MN) was used as the protein source. The protein content of the WPI powder, 92.5% w/w, was determined by microKjeldahl analysis with a conversion factor of $6.38N = \%$ protein.[34] All other chemicals were of reagent-grade quality.

Preparation of WPI Suspensions. The WPI was hydrated to ca. 1.1 times the final concentration by stirring for 1 h in 25−1000 mM NaCl aqueous solutions. The suspensions were degassed in a vacuum chamber connected to an aspirator for 1 h to remove all visible air bubbles, adjusted to pH 7.0 with 1.0 M NaOH or 1.0 M HCl, diluted to the desired protein concentration with an appropriate NaCl solution, and then degassed for an hour.

Dynamic Small-Strain Rheological Measurement. A Bohlin VOR rheometer (Bohlin Reologi, Inc., Cranbury, NJ) was used to determine the rheological transitions which occurred during in situ thermal processing of the WPI suspensions. The Bohlin C14 concentric cylinder measuring system was used in all experiments. The measuring system consisted of a oscillating cup and a fixed bob attached to a 13.2 or 42.2 g•cm torque bar. The WPI suspensions were carefully poured into the cup at 25 °C and covered with a thin layer of mineral oil to prevent water evaporation during the experiments. Gels were formed by heating the samples from 25 to 90 °C at a constant rate of 2.5 °C/min, holding at 90 °C for 1 h. To minimize damage on the gel network and to ensure the linear viscoelastic behavior of the samples, the testing frequency and strain during the gelation process were selected based on the preliminary experimental results from frequency and strain sweeps of the thermally formed gels. A frequency of 0.05 Hz and a maximum strain of the 0.01 were used to measure the storage moduli (G'), loss moduli (G''), and phase angles (δ) of the samples during the entire thermal treatment. The strain sweep measurements up to a strain of 0.2 were done at the end of the temperature holding at 90 °C at a frequency of 1 Hz.

Large-Strain Torsional Test. The response of the gels to strain larger than 0.2 was investigated using a torsional test method. WPI suspensions were poured into glass tubes (19 mm internal diameter) which were precoated with silicon (Sigmacote, Sigma) and closed at one end with rubber stoppers. Gels were formed by heating in a 90 °C water bath for 1 h. After cooling 1 h at room temperature, 25 ± 3 °C, gels were held overnight at 4 °C. The following day gels were equilibrated to room temperature for large-strain testing. Gels were cut into 29 mm long cylinders, each end of which was glued to plastic disks using cyanoacrylate glue. The cylinders were ground on a precision milling machine (Model GC-TG92 US, Gel Consultants, Raleigh, NC) into capstan shapes with a center diameter of 10 mm as described by Montejano et al.[35] Gels were vertically mounted and twisted to fracture at 2.5 rpm on a Hamann torsion gelometer (Gel Consultants, Raleigh, NC). The values of the true shear strain were calculated from the torque and angular displacements as described by Diehl et al.[36]

Evaluation of Fractal Dimension (*d*) from Rheological Data. The volume fraction of particles (ϕ, v/v) in the gels was assumed to be proportional to the protein concentration (C, % w/v). The fractal dimension (d) of gels in the strong-link regime was then evaluated from the slope of the log G' versus log ϕ plot, using eq 2. The value of x in eq 2 was assumed between 1.0 and 1.3[17] because these values have already been confirmed to be valid for the globular proteins used in this study.[10,13,14]

Results

Storage Modulus. During the thermal treatment, the storage modulus, loss modulus, and phase angle were recorded. However, both G' and G'' developed similarly and the G' values were considerably greater in magnitude than the G'' values for all gels, suggesting predominantly elastic behavior. Therefore, only G' values are discussed. Figure 1 shows examples of gelation curves at 90 °C at various NaCl concentrations. In all conditions, G' growth during heating was well-approximated by the first-order reaction kinetics (solid curves), which is consistent with our previous results.[37] First-order reaction kinetics are often observed in gelation of various protein systems.[38−42] Holding at 90 °C for an hour was sufficiently enough to complete the heat-induced aggregation of whey proteins. The small deviation between the measured values of G' at the end of heating and the predicted values at an infinite time based on first-order reaction kinetics (×) did not affect the results in this study.

The values of G' at the end of the heating at 90 °C for an hour are shown in Figure 2. A maximum value of G' was observed at 100 mM NaCl, which is consistent with our previous results.[37] At sufficiently low salt concentrations, electrostatic repulsive forces due to charges on the

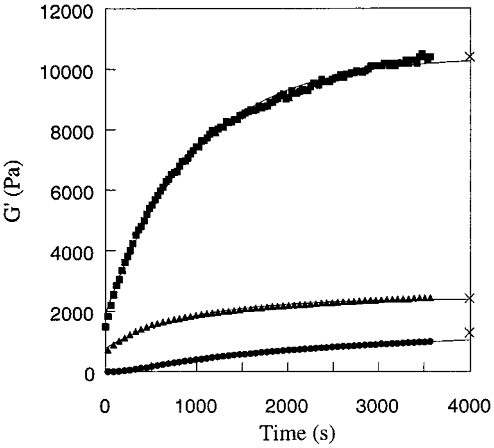

Figure 1. G' development at 90 °C for 10% w/v protein samples formed at ionic strengths of 25 mM (circle), 100 mM (square), and 500 mM (triangle). Solid lines and crosses represent the first-order reaction kinetics and predicted G' values at an infinite time, respectively.

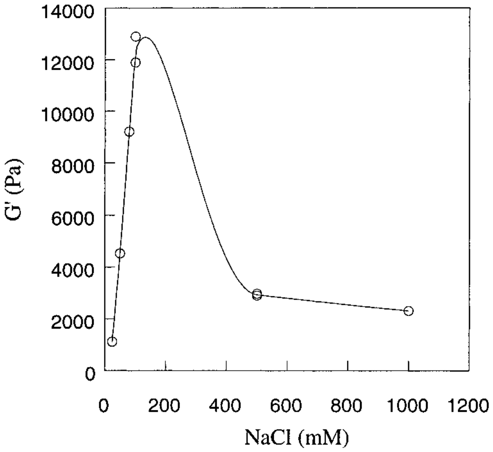

Figure 2. Relationship between ionic strength and final G' values at 1 Hz of 10% w/v protein gels.

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protein molecules are considered to dominant. With increasing salt concentration, attractive interactions become stronger, resulting in an increase in the gel rigidity. Further increases in the salt concentration cause attractive interactions to dominate repulsive ones. Aggregation becomes increasingly random and gel rigidity decreases, resulting in maximum G' values at an intermediate salt concentration around 100 mM.[24]

Strain Sweep. When the applied strain was increased up to 0.2 during the dynamic rheological measurement, the storage modulus remained constant, suggesting no breaking occurred. The typical relationship between shear stress and strain when larger strain was applied is shown in Figure 3a. At large strains, the relationship between strain and stress was not linear: stress/strain values appeared to increase with increasing stress, suggesting that structural changes occurred with increasing strain. Fracture occurred at a relatively large strain (~1–2) which is consistent with our previous results.[43] The strain at fracture (fracture strain) decreased with increasing protein concentration (Figure 3b), suggesting that the breaking occurred in the flocs (the strong-link regime).[16,17] Gels with 25 mM NaCl showed a stronger concentration dependence of the stress at fracture (fracture stress) than gels with 100 or 500 mM NaCl (Figure 3c) due to higher fracture strains (Figure 3b) and strain-hardening effects (Figure 3a).

Fractal Dimension. Typical data for the concentration dependence of G' are shown in Figure 4. All the samples showed power law relationships between G' and the protein concentration (C). A power law behavior of the modulus of whey protein gels has often been reported,[10,13,14,44–49] with exponent values varying from 2 to 7, depending on the experimental conditions. At 25, 100, and 500 mM NaCl, the slope of the double-logarithmic plots of G' and C were 5.4, 2.7, and 3.4, and the evaluated fractal dimension values were ca. 2.2, 1.5, and 1.8, respectively.

Figure 5 summarizes the fractal dimensions of gels with varying NaCl concentrations. At 25 mM NaCl, the value of the fractal dimension (~2.2) agreed with that for the reaction-limited cluster–cluster aggregation process.[33] Further increasing the NaCl concentration decreased the fractal dimensions. The values of the fractal dimension (~1.8), which agreed with those for diffusion-limited cluster–cluster aggregation,[33] were obtained at 50, 80, 500, and 1000 mM. At 100 mM NaCl, a remarkably low value of the fractal dimension (~1.5) was observed.

Discussion

Fractal Nature of the Gels. The values of the fractal dimension evaluated using scaling relationships between G' and ϕ (eq 2) agreed with those evaluated for reaction-limited and diffusion-limited aggregation[33] at low and high NaCl concentrations, respectively (Figure 5). Lin et al. investigated the aggregation of gold colloids and concluded that, if the amount of the surface charge on particles is reduced sufficiently, every collision results in the particles sticking, d taking the value of diffusion-limited aggregates.[50] If a large surface charge exists, many collisions must occur before the particles stick together because of electrostatic repulsion, d taking the value of reaction-limited aggregates.[50] The results in Figure 5 suggest that, at ionic strengths ≥ 50 mM, the surface charge of protein molecules was sufficiently shielded, while at 25 mM NaCl, frequent collisions of proteins were necessary before

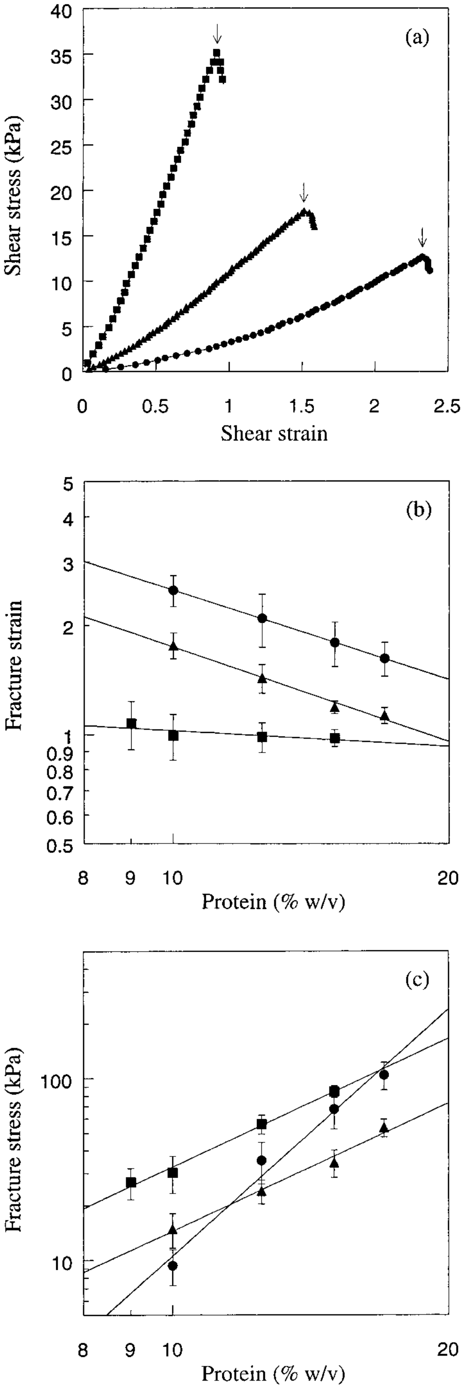

Figure 3. (a) Strain dependence of shear stress values of gels containing 10% w/v protein. Ionic strength: 25 mM (circle); 100 mM (square); 500 mM (triangle). Arrows indicate the fracture point. (b) Protein concentration dependence of fracture strain values of gels formed at ionic strengths of 25 mM (circle), 100 mM (square), and 500 mM (triangle). (c) Protein concentration dependence of fracture stress values of gels formed at ionic strengths of 25 mM (circle), 100 mM (square), and 500 mM (triangle).

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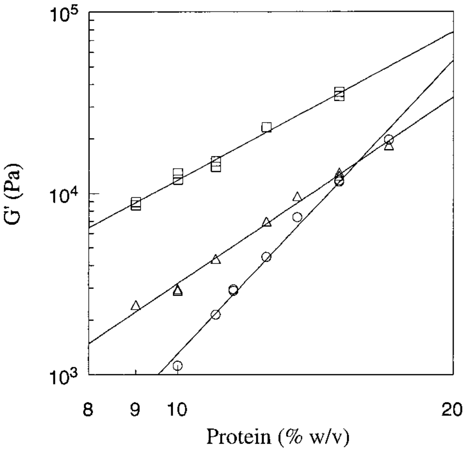

Figure 4. Protein concentration dependence of G' values at 1 Hz of gels formed at ionic strengths of 25 mM (circle), 100 mM (square), and 500 mM (triangle).

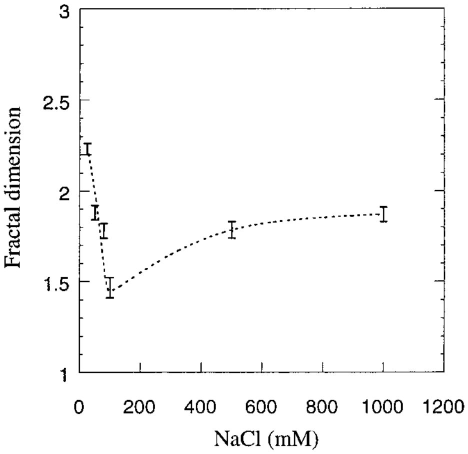

Figure 5. Effect of ionic strength on fractal dimension of WPI gels. Upper and lower limits of each bars represent evaluated fractal dimension values assuming $x = 1.3$ and 1.0, respectively.

forming aggregates due to electrostatic repulsion among the proteins. Since the predominant protein component, β-lactoglobulin, has 10 negative charges per molecule at pH 7,[51] the total amount of charges on the surface of the protein molecules in the examined systems is estimated to be around 50 mM. It is worth noting, however, that, to our knowledge, there is no published result of other techniques such as microstructural images or scattering analyses showing fractal or nonfractal structures of gels formed at low NaCl concentrations (e.g., ≤50 mM). Therefore, the true structure of these gels is not clear.

Verheul et al. reported the scaling exponent of 5 in the relationships between G' and ϕ for WPI gels formed at 68.5 °C in 0.4−3.0 M NaCl.[47] Using this exponent value and eq 2 yields a fractal dimension of ca. 2.2, which agrees with the reaction-limited behavior. In this gelling condition, the denaturation rate is considered low because the denaturation temperature of whey proteins is usually observed around 75 °C using differential scanning calorimetry.[52] Therefore, the aggregation probability is considered low even in high ionic strength (0.4−3.0 M), resulting in a reaction-limited aggregation. Gimel et al. also reported the reaction-limited aggregation ($d \sim 2.0$) of β-lactoglobulin at 70−76 °C based on light-scattering methods.[11] The d value for gels at 25 mM (∼2.2) (Figure 5) suggests that the gelation process at this low ionic concentration was a reaction-limited process, while the value is slightly higher than that evaluated by Gimel et al.[11] This deviation would arise from the complexity of the mixed protein systems. For example, the value of the fractal dimension will be larger if restructuring of aggregates occurs.[33]

A considerably low value of the fractal dimension (∼1.5) was obtained at 100 mM NaCl (Figure 5), indicating the weak applicability of the rheological model. Almost constant values of the yield strain (Figure 3b) also indicate that the gels do not show typical strong-link behavior at this salt concentration. At around 100 mM monovalent ions, the microstructures of the protein gels drastically change from fine strands to particulate aggregates. The microscopic images of protein gels consist of mixtures of fine strands and spherical aggregates at 100 mM.[24] Therefore, a heterogeneous structure formed at 100 mM NaCl may account for the unusual rheological behavior of the gels. In addition, β-lactoglobulin binds NaCl at neutral pH,[53] which may change the charge distribution on protein surfaces. It would be possible that a unique charge distribution on the protein surface causes a low value of the fractal dimension because aggregate particles with anisotropic repulsive interactions are known to tend not to branch, resulting in a straightened stringlike network with a low fractal dimensionality.[54]

Limit of Linearity. Shih et al. predicted a scaling relationship between the volume fraction of particles (ϕ) and the limit strain of linearity (γ_0) of strong-link gels with assumptions that a constant limiting force, which is independent of ϕ, is needed to break the bond:[17]

$$\gamma_0 \propto \phi^{-(1+x)/(3-d)} \tag{3}$$

In the study of Shih et al.,[17] a constant value of G' was observed up to γ_0, at which the weakest bond breaks and thus γ_0 is considered the same as the yield strain. The yield strain value at which fracture occurs is typically less than 0.1 for colloidal gels[17,55] or larger than 2 for polyacrylamide gels,[56] while both gels show linear viscoelasticity where stress and strain are proportional up to the fracture point. For protein gels, however, fracture usually occurred outside the linear viscoelasticity region (Figure 3a), suggesting that the limit of linearity is not determined by breaking of the weakest bonds but rather by structural changes. The strain hardening behavior observed in this study can be explained if the stress-carrying strands in the flocs extend as the strain increases. The values of the fractal dimensions calculated using the relationship between fracture strain and concentration (Figure 3b) with eq 3 are unreasonable, (e.g., for gels with 25 and 500 mM NaCl, $d = 0.2{-}0.6$ and $0.3{-}0.7$, respectively). Therefore, it seems that γ_0 is less dependent on ϕ than predicted by eq 3, probably because the limiting force necessary for breaking of the weakest bonds increases with ϕ. However, since eq 3 was independently derived from eq 2, inapplicability of one equation does not mean invalidity of another.

Relevance with Rheological Studies. Salt addition changes the balance between repulsive and attractive forces among proteins, which will have several consequences. With increasing ionic strength, the strands of

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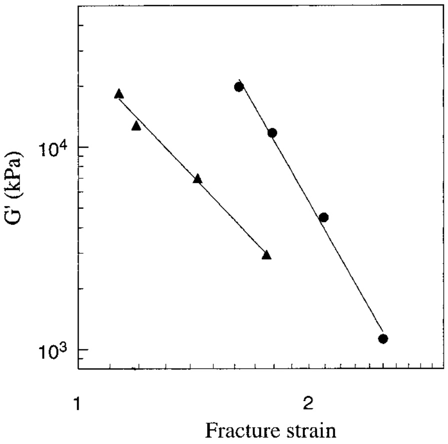

Figure 6. Relationship between fracture strain and G' values at 1 Hz of gels formed at ionic strengths of 25 mM (circle) and 500 mM (triangle).

proteins are considered to become thicker due to reducing repulsive forces and thus increasing attractive forces among the proteins. An increase in the thickness of the strands implies an increase in K_0 in eq 1, which may be responsible for an increase in G' with NaCl up to 100 mM (Figure 2). Further increasing the ionic strength causes more random aggregation, leading to a larger size of aggregated particles.[12,20,21] Small-angle neutron scattering experiments have revealed that the size of the building flocs of the gel network becomes larger especially at ionic concentrations > 100 mM.[57] This coincides with a larger water permeability of gels with increasing salt concentration.[48] G' thus decreases due to large flocs with low elasticity (eq 1) when the salt concentration is >100 mM.

Large-strain (fracture) properties reflect differences in the gel structures which are not detected by small-strain techniques. The small-strain measurements in the linear viscoelasticity region give similar low values of G' both for fine-stranded gels formed at low ionic strength and for particulate gels formed at high ionic strength (Figure 2), while fine-stranded gels usually fracture at a higher strain than particulate gels (Figure 3b).[24,26] Figure 6 shows the relationship between G' and the fracture strain for the gels with 25 and 500 mM NaCl. For the gels with comparable values of G', fracture strain values would decrease with increasing ionic strength. Since the fracture strain depends on the extent to which a macroscopic shear strain leads to a deformation of the particles and bonds (ξ/L),[58] in the case that the elasticity of the flocs is comparable, gels consisting of smaller flocs are considered more strain resistant. In addition, the number of flocs in the gels increases with decreasing floc size. It is therefore reasonable that fine-stranded gels with smaller flocs are more strain resistant than particulate gels even if G' values are comparable. Although this speculation is only a first-order approximation, the rheological model with the fractal concept used in this study is satisfactorily in harmony with the known rheological behavior of whey protein gels. Quite recently, a direct relationship was found between the fractal dimension of a biopolymer gel and the time at which the structure fails when a constant force is applied to the gels.[59]

Conclusions

A rheological analysis is one of the few methods that is useful to analyze structures in globular protein gels with high protein content. All the gels formed from a mixture of globular proteins at various ionic strengths showed a power law relationship between the storage modulus and protein concentration, consistent with previous results for globular protein gels having fractal structures. The ionic strength dependence of the fractal dimension suggested that the probability of the protein aggregation on collision rapidly increased at ionic strengths around 25–50 mM, which agreed with the total amount of charged groups on the proteins. Above an ionic strength of 50 mM, the values of the fractal dimension remained almost constant except for gels with 100 mM NaCl. Consequently, changes in the storage modulus with increasing ionic strength are considered mainly due to an increase in the size of flocs building gel networks and a thickening of strands in the flocs. Aggregation process of globular proteins can be treated in analogy with that of colloidal particles.

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