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Analysis of Aggregate Structure in Food Protein Gels with the Concept of Fractal

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The fractal structure of the aggregates in food protein gels was analyzed. Three kinds of food protein gels were prepared: (1) β-lactoglobulin (β-LG) gel; (2) 11S soybean globulin gel; and (3) caseinate gel. From the concentration dependence of the gel elasticity, the fractal dimensions D_f of the aggregates in the gels were evaluated, according to the theory of Shih *et al.* These gels showed the weak-link behavior described in the theory of Shih *et al.* The values obtained for D_f were 2.6–2.7, which were larger than those predicted by the cluster-cluster aggregation model for a dilute system. In addition, for the β-LG gels, the fractal dimension was also evaluated from the analysis of the gel image obtained with a confocal scanning laser microscopy, the value being close to that evaluated from the concentration dependence of the gel elasticity. These results indicate that the elastic behavior of the aggregate gels is a reflection of fractal structure of the aggregates in the gels.

Key words: fractal; confocal scanning microscopy; elasticity; gel; aggregate

Heating or acidifying a globular protein solution causes aggregates of protein molecules by interactions such as hydrophobic interaction, disulfide bond formation, or electrostatic interaction, and the gel is often formed at a high concentration.[1] Many studies have reported that such macroscopic properties as elasticity of aggregate gels for many proteins vary with the aggregation conditions; *e.g.*, pH and ionic strength.[2–4] In these studies, however, only the correlation between the conditions and the macroscopic physical properties has been repeatedly discussed. To systematically understand the behavior of the macroscopic physical properties of protein gels, the relationship between the structure of the aggregates and the macroscopic physical properties should be investigated. However, the aggregates in protein gels were difficult to characterize due to their disordered shape.

Recently, fractal analysis has attracted attention as a quantitative analytical method that can characterize many kinds of disordered shapes.[5] A fractal is a self-similar structure that can be characterized by a non-integer dimension; the fractal dimension D_f.[5,6] It has been shown that the colloid aggregate formed in dilute systems such as gold and silica can be characterized as a fractal.[7,8] On the other hand, the studies on the fractal structure of gels formed by aggregation has not been well done, especially on proteins. Bremer *et al.* examined the fractal structure of caseinate gel prepared by addition of glucono-δ-lactone from the concentration dependence of the elasticity of the gel.[9–11] Shih *et al.* evaluated the fractal dimension of boehmite alumina colloidal gels from the measurement of the elasticity of the gels, using a theory different from that of Bremer *et al.*[12]

We have shown that the value of the fractal dimensions of the aggregates formed by heating dilute bovine serum albumin (BSA) solutions was 1.8 or 2.1 with light scattering methods, being close to those predicted by the cluster-cluster aggregation model.[13] We have also done fractal analysis of the aggregates in BSA gels by two different methods: one is by measurement of the concentration dependence of gel elasticity using theory of Shih *et al.*, the other is by analysis of the images obtained with confocal scanning laser microscopy.[14] Consequently, it was concluded that the values of the fractal dimensions obtained from the rheological measurement agreed with those from the image analysis, indicating that the elastic behavior of the BSA gels is a reflection of the fractal structure of the aggregates in the gels. However, such approach has not been attempted for other food protein gels.

In this study, as typical food proteins, β-lactoglobulin gel (heat-induced gel), 11S soybean globulin gel (induced by addition of glucono-δ-lactone and heating), and caseinate gels (induced by addition of glucono-δ-lactone) were chosen and the fractal structures of the gels were analyzed.

Theoretical

Theory of Shih et al.[12]

The structure of colloidal gel (aggregate gel) is approximated as closely packed fractal flocs, and the elastic property of the gel is dominated by that of the flocs.[12] Depending on the strength of the links between the neighboring flocs in comparison with that in the flocs, the links are classified into two types; strong-link and weak-link. In the strong-link regime, the links between the neighboring flocs have a larger elasticity than those in the flocs. For the gel with a strong-link (hereafter referred to as a strong-link gel), the dependence of the elasticity E and the limit of linearity γ_0 of the gels on the particle (in this study, protein) concentration ϕ can be described as follows:

$$E \sim \phi^{(3+x)/(3-D_f)} \quad (1)$$

$$\gamma_0 \sim \phi^{-(1+x)/(3-D_f)} \quad (2)$$

where D_f is the fractal dimension of the flocs ($D_f \leq 3$), and x is the backbone fractal dimension of the flocs, which varies

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between 1.0 and 1.3.[12] On the other hand, in the weak-link regime, the links in the flocs have a larger elasticity than those between the neighboring flocs: for the gel with a weak-link (hereafter referred to as a weak-link gel), the dependence of the E and γ_0 on particle concentration ϕ can be expressed as follows:

$$E \sim \phi^{1/(3-D_f)} \tag{3}$$

$$\gamma_0 \sim \phi^{1/(3-D_f)} \tag{4}$$

Materials and Methods

Materials. β-Lactoglobulin (β-LG) was obtained from Sigma Chemical Co. (St. Louis, ref. L-6879) and was a mixture of genetic variants A and B. 11S soybean globulin was isolated from defatted soybean meal, the details of the isolation methods being described by Nagano *et al.*[15] Caseinate was purchased from Sigma Chemical Co. (ref. C-8654). All other chemicals were of reagent grade.

Preparation of gels for elasticity measurement. The gels for the elasticity measurement were prepared as follows.

(1) *β-LG gels.* β-LG was dissolved in 50 mM HEPES buffer (pH 7.0, NaCl was added to make the ionic strength of buffer 1.0 M). The solutions were degassed under a vacuum for 3 min. The pH of the solutions was then adjusted to the pH of the buffer, using NaOH solutions. The β-LG solutions prepared above were poured into glass tubes (28 mm in outer diameter, 20 mm in inner diameter, 40 mm in height) coated with Sigmacoat (Sigma Chemical Co., St. Louis, U.S.A.) and each end was closed with a Teflon cap. The samples were preheated at 40°C for 60 min to prevent the production of bubbles and then heated in a water bath at 95°C for 10 min. The samples were immediately cooled to 25°C in a water bath. The gels were removed from the tube and cut into a cylindrical shape (15 mm in height) and stored in silicon oil at 25°C for 24 h before measurement.

(2) *11S Globulin gels.* 11S Globulin gels were prepared similarly to those described by Kohyama *et al.*[16,17] The 11S globulin was dispersed in Milli-Q water (Millipore Co.). The samples were preheated in a boiling water for 3 min. The preheated samples were immediately cooled in an ice bath for 5 min and then degassed under vacuum to remove bubbles. Glucono-δ-lactone (GDL) was then added to the solutions. The weight ratio of added GDL to 11S globulin was 1 : 10.[16,17] The samples were stirred for 1 min at 25°C. Then, sample solutions were poured into glass tubes and each end was closed with a Teflon cap. The samples were heated in a water bath at 70°C for 30 min, and immediately cooled to 25°C in a water bath. The gels were cut into a cylindrical shape (15 mm height) and stored in silicon oil at 25°C for 24 h before measurement.

(3) *Caseinate gel.* Caseinate gels were prepared similarly to those described by Bremer *et al.*[9-11] Caseinate was dissolved in Milli-Q water. GDL was added to the solutions at the ratio of 0.25 g GDL per 1 g caseinate, and samples were stirred for 1 min at 25°C. The sample solutions prepared above were poured into glass tubes, each end being closed with a Teflon cap. The samples were set in a water bath at 25°C. After 2 h, the gels were removed from the tube, and cut into a cylindrical shape (15 mm height) and stored in silicon oil at 25°C for 24 h before measurement.

Measurement of gel elasticity. Gel elasticity was measured by a uniaxial compression test with a Rheoner RE-3305 (Yamaden, Co., Tokyo, Japan) as reported in the preceding study.[14] A cylindrical gel sample was vertically compressed with a polyacetal flat plunger of 30 mm diameter at a compression rate of 1.0 mm/s. The strain γ was calculated as the ratio of the deformation to the initial height of the gels. The elasticity E was calculated from the linear part of the stress–strain curve over a small strain range.

Evaluation of fractal dimension D_f from rheological data. The fractal dimension D_f was evaluated by the method used in the preceding study.[14] First, the link-type of gel was identified from the dependence of γ_0 on ϕ, γ_0 being taken as the strain value where the deviation between the ordinate value of the stress–strain curve F/S and that of $E \times \gamma$ was 5%. Since γ_0 decreases with increasing ϕ for a strong-like gel and increases with increasing ϕ for a weak-link gel (see Eqs. (2) and (4)), the cross-link type for the gel can be identified from the sign of the slope of $\log \gamma_0$ *vs.* $\log \phi$ plot.

The fractal dimension D_f is then calculated from the slope of $\log E$ *vs.* $\log \phi$ plot, using Eq. (1) for a strong-link gel and Eq. (3) for a weak-link gel.

Image analysis of aggregates in gels using confocal scanning laser microscopy. Since the clear image of the aggregates for the 11S globulin gel and caseinate gels were not observed using the confocal scanning laser microscopy, the image analysis was only done for the β-lactoglobulin gel.

(1) *Sample preparation for confocal scanning microscopy.* β-Lactoglobulin gels for the confocal scanning laser microscopy were prepared as follows. The β-LG solution preheated at 40°C for 60 min, as explained before, was poured into a gap of 0.18 mm between two glass plates with a spacer made from Niftron tape (Nitto Denko Co., Osaka, Japan). The samples were heated at 95°C for 10 min. Thereafter, the sample was cooled at 25°C. After 24 h, the gel strips were removed and cut into 5-mm squares. The gels obtained were stained with a solution of fluorescein isothiocyanate (FITC), a fluorescent labeling agent for proteins.[18] For the staining solution, buffer containing 0.001 wt% FITC was used. The gels were immersed in the staining solution for 1 h with gentle shaking and subsequently, washed in fresh buffer for 1 h. The stained gels were then mounted on a slide glass with spacer around the gels, and a cover glass was placed on the top of the spacers. After the cover glasses were fixed with clear nail polish, this sample was used for confocal laser scanning microscopy observation.

(2) *Confocal scanning laser microscopy.* A confocal laser scanning microscope model MRC600 (Bio-lad Laboratories, Inc., CA, U.S.A.) was used.

(3) *Evaluation of fractal dimension D_f from the images of protein aggregates in a gel.* The same procedures as that in our preceding study[14] were used. The obtained confocal microscopy images were digitized with the public domain NIH Image program ver. 1.59[19] on the Macintosh platform. From the digitized image, the fractal dimension D_f was calculated by the box counting method[20]; a brief procedure for the method is as follows[21]:

[1] A square mesh of a certain size L is laid over the object on the digitized image.

[2] The number of mesh boxes $N(L)$ that contain part of the image is counted.

[3] The fractal dimension of the protein aggregates on the image, D, is calculated from the slope of the double logarithmic plot for $N(L)$ *vs.* L, considering the relationship between the parameters:

$$N(L) \sim L^{-D} \tag{5}$$

The fractal dimension of protein aggregates of three dimensions D_f can be calculated from the following equation[6]:

$$D_f = D + 1 \tag{6}$$

Computer software for fractal analysis based on the box counting method[21] was used in this study.

Results

Figure 1 shows stress–strain curves for the β-lactoglobulin gels (A), 11S globulin gels (B), and caseinate gels (C).

Figure 2(A) shows the double logarithmic plot of the limit of linearity γ_0 *vs.* β-lactoglobulin concentration ϕ for the β-lactoglobulin gels. Since γ_0 tended to increase with increasing ϕ, this gel is confirmed to be a weak-link gel, as explained before. Figure 2(B) shows the double logarithmic plot of the gel elasticity E *vs.* ϕ for the same samples as those in Fig. 2(A). From the slope of the plot, using Eq. (3) for weak-link gels, the fractal dimension D_f was evaluated to be 2.62. Figures 3(A) and 3(B) show the double logarithmic plots of γ_0 *vs.* ϕ and E *vs.* ϕ, respectively, for 11S globulin gels. From the slope of the $\log \gamma_0$ *vs.* $\log \phi$ plot (A), this gel is confirmed to show weak-link behavior, and the fractal dimension D_f was evaluated to be 2.70, using Eq. (3). Figures 4(A) and 4(B) are the double logarithmic plots of γ_0 *vs.* ϕ and E *vs.* ϕ, respectively, for caseinate gels. From the slope of $\log \gamma_0$ *vs.* $\log \phi$, this gel can also be confirmed to show weak-link behavior, and the fractal dimension D_f was evaluated to be 2.65, using Eq. (3).

A typical original image taken with the confocal scanning laser microscopy and a digitized one of the β-lactoglobulin

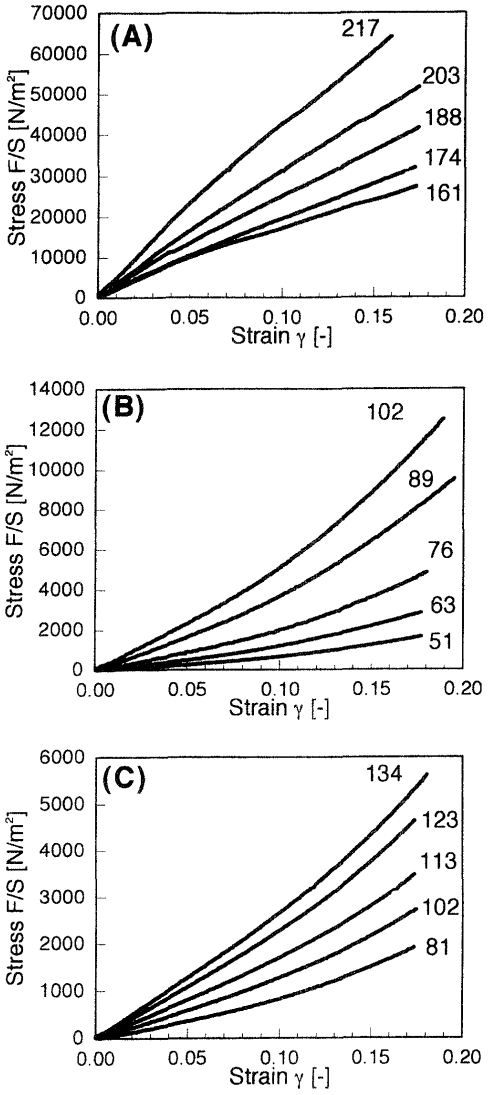

Fig. 1. Stress–Strain Curves for the β-Lactoglobulin (A) and 11S Globulin (B), and Caseinate Gels (C).

The numbers in the figures represent the concentration of the gels (kg/m^3).

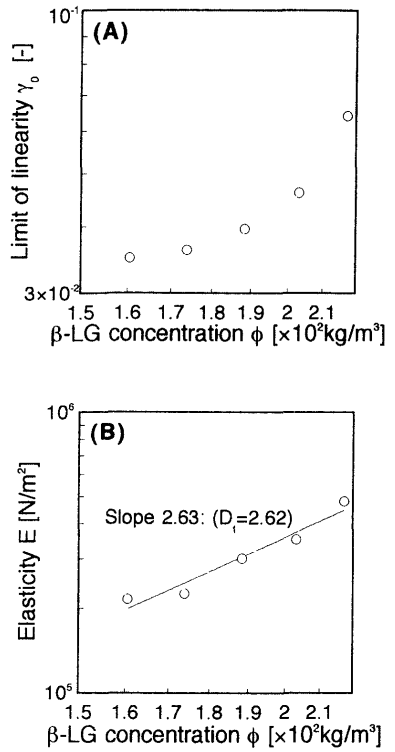

Fig. 2. Double Logarithmic Plots for the Limit of Linearity γ_0 *vs.* β-LG Concentration ϕ (A) and Elasticity E *vs.* ϕ (B).

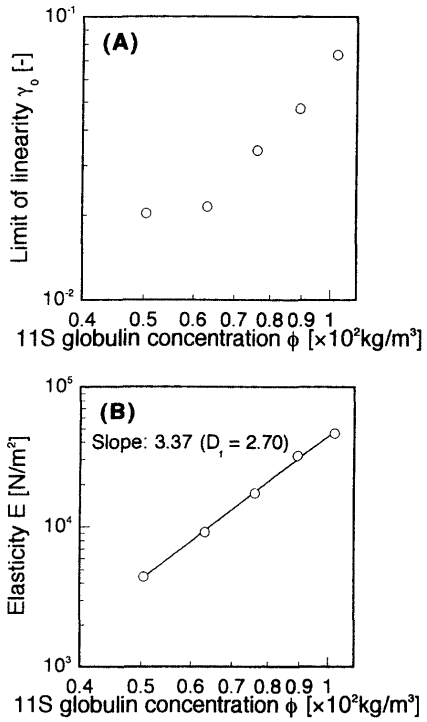

Fig. 3. Double Logarithmic Plots for the Limit of Linearity γ_0 *vs.* 11S Globulin Concentration ϕ (A) and Elasticity E *vs.* ϕ (B).

gel is shown in Figs. 5(A) and (B), respectively. Figure 6 shows the double logarithmic plot of the box number $N(L)$ *vs.* box size L for the β-lactoglobulin gels. The plot shows power law dependence for the box number $N(L)$ on box size L, as predicted by Eq. (5). From the slope of the plot, the value of D in Eq. (5) for the gel was 1.68, the value of D_f being calculated to be 2.68 from Eq. (6). This value of D_f was close to that obtained from the concentration dependence of the gel elasticity. In addition, irrespective of the β-lactoglobulin concentration in the concentration range examined, the values of D_f obtained from the image of the gels were almost constant, though the data are not shown.

Discussion

In this study, by the theory of Shih *et al.*, the fractal dimension of the aggregates in the gel was obtained for three kinds of protein gels. In addition, as for the β-LG gels, from the image analysis, the value of the fractal dimension of the aggregates in the gel was also evaluated, and their value was close to that obtained from the elasticity measurement. In the preceding study, we have found that the values of D_f for the aggregates in the BSA gels obtained from the image analysis were also close to those evaluated from the elasticity measurement.[14] From these aspects, it is suggested that the elastic behavior of the aggregate gels is a reflection of the fractal structure of the aggregates in the gels, irrespective of proteins.

For caseinate gels, from the concentration dependence of the gel elasticity, Bremer *et al.* also evaluated the value of the fractal dimension for the aggregates in the gels by using their theory.[9–11] In their theory, they classified gels into two types; type 1 gel and type 2 gel. For a type 1 gel,

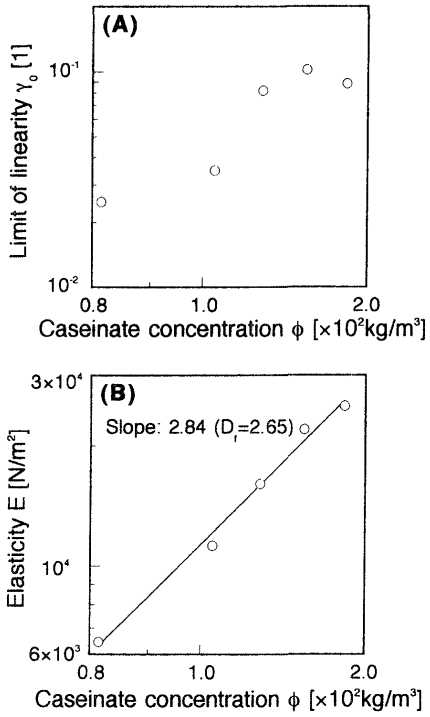

Fig. 4. Double Logarithmic Plots for the Limit of Linearity γ_0 *vs.* Caseinate Concentration ϕ (A) and Elasticity E *vs.* ϕ (B).

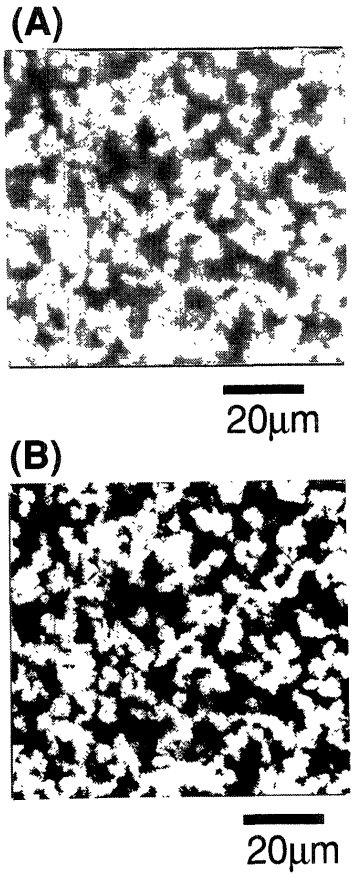

Fig. 5. Photographs Obtained Using a Confocal Scanning Laser Microscopy for the Aggregates in the β-LG Gel.

(A): An original image. (B): A digitized image Sample: β-LG gel (concentration; 161 kg/m^3) prepared with 50 mM HEPES buffer (pH 7.0, NaCl was added to make the ionic strength of the buffer 1.0 M) as a solvent.

the strands composing the network in the gel are stretched or shrunk under applied stress, while the strands of the type 2 gels are bent under applied stress. Equations for evaluating

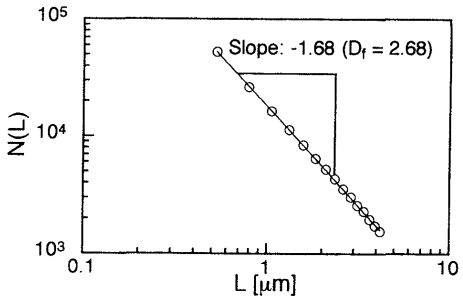

Fig. 6. Plot for Evaluation of Fractal Dimension D_f for the Aggregates in the β-LG Gel.

the fractal dimensions are diffeent between the two types of gel. It is, however, difficult to identify experimentally such strand properties and therefore to decide which equation in their theory should be used. Consequently, from the theory of Bremer *et al.*, one can only guess the types of a gel (type 1 or type 2), by comparing the value of the fractal dimension obtained with that evaluated by another method such as image analysis. On the other hand, the cross-link type in the theory of Shih *et al.* is identified in principle from the dependence of γ_0 on ϕ, as explained before. Thus, the fractal dimension D_f can be measured from rheological measurement only, the theory of Shih *et al.* used in this study having this advantage over that of Bremer *et al.*

The stress–strain curves for the 11S globulin gels and caseinate gels bent upwards. We have shown that those for the BSA gels also curved upward in our preceding study.[14] On the other hand, those of β-LG gels curve downwards. This difference suggested that the mechanism for causing nonlinearity of the elasticity (for example, breaking bonds) was different between β-LG gels and the other gels. Tracing the deviation of the theory of Shih *et al.*, however, it is found that Eqs. (1)–(4) in this study are derivable without supposing the specific mechanism for causing nonlinearity of the elasticity. Therefore, the equations are applicable, independent of the mechanism for causing nonlinearity. In addition, not only for the β-LG gels in this study (the stress–strain curves bent downwards) but also for the BSA gels in the preceding study (the stress–strain curves bent upwards), the values of D_f obtained from the elasticity measurement were close to those evaluated from the image analysis, demonstrating the applicability of the theory of Shih *et al.*, irrespective of the shape of the stress–strain curves.

According to the computer simulation of the aggregation process that diffusing particles and aggregates in certain medium stick to each other on contact, the values of the fractal dimension for the aggregate vary from 1.8 to 2.1.[6,22,23] For the aggregates in the gels of alumina colloid, Shih *et al.* also obtained a similar value ($\approx$2.1) from the elasticity measurements.[12] On the other hand, the values of the fractal dimensions of the gels prepared in this study were about 2.6 to 2.7. Moreover, in the preceding study, we have shown that the value of the aggregates in the BSA gels were approximately 2.8.[14] These values were larger than those calculated by the computer simulation (1.8 or 2.1). Meakin *et al.* reported that the value of the fractal dimension for the aggregates on the simulation considering the restructure of each aggregate was larger than that

calculated by neglecting the restructuring.[23,24] It is suggested that the larger values of the fractal dimension for protein gels are caused by such restructuring.

Shih *et al.* reported that boehmite alumina colloidal gels showed the behavior of the strong-link.[12] In the preceding study, we observed the weak-link behavior for the BSA gels.[14] However, there is no other study that observes the weak-link behavior from the dependence of the limit of linearity for gels on gel concentration. In this study, it was shown that the aggregate gels prepared above were also weak-link gels.

As for the 11S globulin and caseinate gels, from the confocal scanning laser microscopy, the clear image of the aggregates were not obtained, suggesting that the size of the aggregates was smaller than the smallest size of which the confocal scanning microscopy used can detect. To analyze the structure of the aggregates for these gels, another experimental method rather than the confocal scanning microscopy should be developed.

In conclusion, the β-LG gel, 11S soybean globulin gels, and caseinate gels prepared in this study showed the weak-link behavior in the theory of Shih *et al.*, and from the concentration dependence of the gel elasticity, the value of the fractal dimension could be evaluated, by the theory of Shih *et al.* In addition, as for the β-LG gels, from the image analysis the fractal dimension could be also obtained, the value being similar to that evaluated from the concentration dependence of the gel elasticity above.

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