HYDROCOLLOIDS – PART 1
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Evaluation of the fractal dimension for aggregates in heat induced BSA gels

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The fractal structure of aggregates in heat-induced BSA gels prepared with and without salt ($CaCl_2$ or NaCl) was examined. From the concentration dependence of the gel elasticity determined from a uniaxial compression test of the gel, the fractal dimensions D_f of the aggregates in the gels were evaluated, using the theory of Shih *et al.*. It was confirmed that the gels with and without salt showed weak- and strong-link behavior in the theory of Shih *et al.*, respectively. The obtained values of D_f were about 2 for strong-link gels and about 2.7 for weal-link gels. In addition, as for the gels containing salt (weak-link type), from the analysis of the gel image obtained with confocal scanning laser microscopy of the gels, the fractal dimensions were also evaluated, the value being close to that evaluated from the gel elasticity measurements. These results indicate that the elastic behavior of the aggregate gels is a reflection of fractal structure of the aggregates in the gels.

1. INTRODUCTION

The aggregate structure in heat-induced protein gels would influence the macroscopic physical properties for gel. Many studies have been reported in which the macroscopic properties of aggregated protein gels vary with the aggregation conditions; *e.g.*, pH and ionic strength; however, only the correlation between the conditions and the macroscopic physical properties has been repeatedly discussed so far. 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.

Recently, fractal analysis has attracted attention as a quantitative analytical method that can characterize many kinds of disordered shapes [1]. A fractal is a self-similar structure which can be characterized by a non-integer dimension; the fractal dimension D_f [1,2].

In this study, the fractal structure of aggregates in heat-induced bovine serum albumin (BSA) gels was examined.

2. MATERIALS AND METHODS

2.1. Theory of Shih *et al.* [3]

The structure of a colloidal gel (aggregated gel) is approximated as closely packed fractal flocs, and the elastic property of the gel is dominated by that of the flocs [3]. 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 higher 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 (γ_0 is the upper limit value of strain (γ) where the stress σ is proportional to γ) of the gels on the particle (in this study, protein) concentration ϕ can be described as follows:

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

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

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 between 1.0 and 1.3 [3]. On the other hand, in the weak-link regime, the links in the flocs have a higher 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:

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

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

2.2. Preparation of Gels for Elasticity Measurement

BSA (Boehringer Mannheim GmbH, Mannheim, Germany; ref.238040) was dissolved in the four kinds of buffer; (a) 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanes ulfonic acid) buffer (pH 7.0, no salt was added), (b) 50 mM acetate buffer (pH 5.1, NaCl was added to make the ionic strength of the buffer 0.1 M), (c) 50 mM HEPES buffer (pH 7.0, $CaCl_2$ was added to be 30 mM), and (d) 50 mM HEPES buffer (pH 7.0, $CaCl_2$ was added to be 5 mM). The solutions were degassed under vacuum for 3 min to remove air. The pH of the solutions was then adjusted to the pH of the buffers, using NaOH and HCl solutions.

2.3. Elasticity Measurements

The elasticity of the gels was determined from an uniaxial compression test with Rheoner RE-3305 (Yamaden, Co., Tokyo, Japan). A cylindrical gel was vertically compressed with a flat plunger (30 mm diameter) at a compression rate 1.0 mm/s. The strain γ of the gel was determined as the ratio of the deformation to the initial height of the gel. The elasticity E was calculated from the linear part of the stress-strain curve at $\gamma < 0.01$.

2.4. Evaluation of the Fractal Dimension from Elasticity Measurements

First, γ_0 was plotted against ϕ. Because γ_0 decreases with increasing ϕ for a strong gel and increases for a weak-link gel as shown in Eqs (2) and (4), the link type for the gel can be identified from the sign of the slope of the plot for $\log\gamma_0$ *vs.* $\log\phi$. As the limit of linearity γ_0, the strain value where the deviation was 5 % between the ordinate value of the stress-strain curve σ and γE was taken.

For the weak-link gels, the value of the fractal dimension D_f is evaluated from the slope of the logE *vs.* $\log\phi$ plot, using Eq. (3) for a weak-link gel. For the strong-link gel, to determine the value of D_f from Eq. (1), the value of the fractal dimension for the effective backbone of the aggregate x is necessary. However, because x took a value from 1.0 to 1.3 [2], the obtained value of D_f varies little in the range of the value of x=1.0～1.3. Therefore, in this study, the minimum and maximum values of D_f were estimated from the slope of the logE *vs.* $\log\phi$ plot, using the value of x as 1.0 to 1.3, for a strong-link gel.

2.5. Evaluation of fractal dimension D_f from the images of protein aggregates in a gel

Samples were heated between two glass plates (a gap is 0.18mm) at 95°C for 10 min. Thereafter, the sample was cooled to 25°C and stored for 24h. The gel strips were removed and cut into 5 mm squares. The obtained gels were immersed in the buffer containing 0.001 wt% fluorescein isothiocyanate (a fluorescent labeling agent for proteins) for 1h with gentle shaking and subsequently washed in fresh buffer for 1h. The stained gels were then mounted on a slide glass with a spacer around the gels. Then, a cover glass was placed on top of the spacers and fixed with nail polish. A confocal laser scanning microscope model MRC600 (Bio-Rad Laboratories, Inc., California, USA) was used for observing aggregates in the gel.

The obtained confocal microscopy images were digitized with the public domain NIH Image program ver.1.59 [4] on the Macintosh platform. From the digitized image, the fractal dimension D_f was calculated by the box counting method [5,6] as follows: A square mesh of a certain size L is laid over the object on the digitized image. The fractal dimension of the protein aggregates on the image, D, is determined using Eq. (5) from the slope of the double logarithmic plot for N(L) *vs.* L; computer software for fractal analysis [5] was used.

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

where N(L) is the number of mesh boxes that contain part of the image. The fractal dimension of protein aggregates of three dimensions D_f can be calculated from the following equation [2]:

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

3. RESULTS [7,8]

Figure 1(A) shows the double logarithmic plot of γ_0 *vs.* ϕ for the BSA gels prepared with

the BSA solution of pH 7.0 (containing 30 mM $CaCl_2$). Because γ_0 tended to increase with increasing ϕ, this gel is confirmed to be a weak-link gel, as explained before. Figure 1 (B) shows the double logarithmic plot of E *vs.* ϕ for the same samples as those in Figure 1(A). From the slope of the plot, using Equation (3) for weak-link gels, the fractal dimension D_f was evaluated to be 2.82. These gels had a turbid appearance.

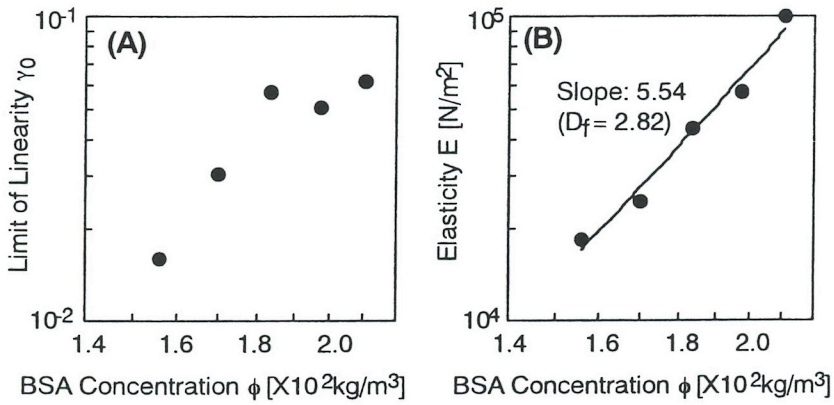

Figure1 The double-logarithmic plots of γ_0 vs. ϕ (A) and E vs. ϕ (B).
Solvent: 50mM HEPES buffer (pH 7.0, 30mM $CaCl_2$)

Figures 2 (A) and 2 (B) show the double logarithmic plots of γ_0 *vs.* ϕ and E *vs.* ϕ, respectively, for the BSA gels prepared with 50 mM HEPES buffer (no salt was added). From the slope of the $\log\gamma_0$ *vs.* $\log\phi$ plot, this gel is confirmed to show a strong-link behavior, and the value of the fractal dimension D_f was estimated to be D_f=2.00~2.07, using Eq.(1). The gel had a transparent appearance. Moreover, aggregates of micrometer order were not observed in the gels using the confocal laser scanning microscope, suggesting that the order of the aggregate size was smaller than a micrometer.

The Table 1 summarizes the results of the fractal analysis of the BSA gels from the elasticity measurements. The value of D_f for the strong-link gel was about 2, while those for the weak-link gels were 2.6-2.8.

Figure 3 shows the double logarithmic plots of the box number N(L) *vs.* box size L for a gel prepared by the same way as that in Figure 1 (BSA concentration was 197 kg/m^3). The plot shows a power law dependence of the box number N(L) on box size L, as predicted by Equation (5). From the slope of the plot, the value of D of the gels was 1.81, the values of D_f being 2.81. This value of D_f was very similar to that obtained from the dependence of the elasticity of the gel on the concentration (Figure 1), indicating that the elastic behavior of this gel is a reflection of the fractal structure of the aggregates in the gels. The obtained value of D_f for this gel was larger than that of the aggregates in dilute BSA solution reported in a previous study [9]. In addition, the value of D_f obtained from the image of the aggregates was almost constant, irrespective of the BSA concentration in the concentration range examined, though the data are not shown.

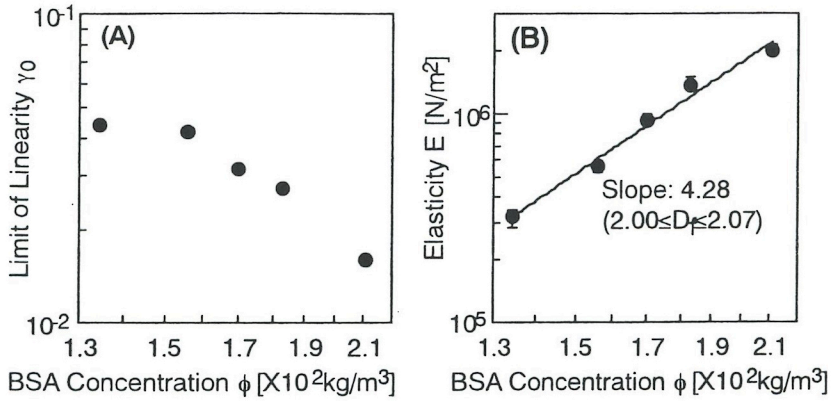

Figure 2 The double-logarithmic plots of γ_0 vs. ϕ (A) and E vs. ϕ (B).
Solvent: 50mM HEPES buffer (pH 7.0, no salt was added)

Table 1 Summary of the fractal analysis of BSA gels prepared with various buffers.

buffer conditions	link type	fractal dimensions D_f
pH 7.0, no salt was added	strong	2.00-2.07
pH 7.0, 30mM $CaCl_2$	weak	2.82
pH 7.0, 5mM $CaCl_2$	weak	2.82
pH 5.1	weak	2.61

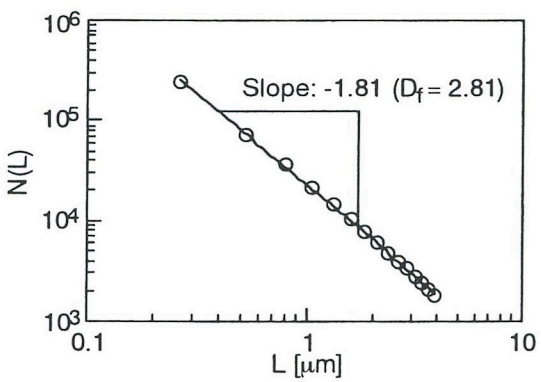

Figure 3 Estimation of fractal dimension D_f by the box counting method.
Buffer: 50mM HEPES buffer (pH 7.0, 30mM $CaCl_2$)
BSA concentration: 197 kg/m^3

4. DISCUSSION

Shih *et al.* [3] reported that boehmite alumina colloid gels showed a strong-link behavior.

However, a gel with a weak-link behavior has not been reported. In the present study, the BSA gel without salt showed a strong-link behavior (Table 1), while the other BSA gels showed a weak-link behavior. That is, it was confirmed that the protein aggregate gels showed both strong- and weak-link behavior by varying the aggregation conditions.

As can be seen from Table 1, the values of D_f for BSA gels with salt were approximately 2.6-2.8, while that of D_f for the gel prepared without salt were about 2. We have already analyzed the fractal structure of aggregates formed by heating dilute BSA solution (BSA concentration, 0.1wt%; ionic strength, 0.1M) with the static light scattering method [9]; the fractal dimension of the aggregates at pH 7.0 (apart from the isoelectric point of BSA (pH, 4.9)) was about 2.1, the value of which agreed with that predicted by the reaction-limited cluster-cluster aggregation model [2,10], while at pH 5.1, the fractal dimension was about 1.8, which agreed with that predicted by the diffusion-limited cluster-cluster aggregation model [2,11]. The results suggested that the amount of average charge of BSA molecules influence the fractal structure of aggregates. Meakin *et al.* [12] stated that the fractal dimension of aggregates increased by "restructuring" during aggregation. The larger values of the fractal dimension for the weal-link gels would also be caused by the restructuring. Salt addition would restructure protein aggregates and make the value of D_f increase.

As for the BSA gel prepared with 50 mM HEPES buffer containing no salt, from confocal scanning laser microscopy, a clear image of the aggregates was not obtained, suggesting that the size of the aggregates was smaller than the smallest size which the confocal scanning microscope used could observe. To analyze the structure of the aggregates for these gels, another experimental method rather than confocal scanning microscopy should be developed.

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